

**The performance of the visual system in relation to retinal structure
and function in an Australian marsupial, the tammar wallaby**

(*Macropus eugenii*)

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University

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Acknowledgements

Abstract

General Introduction

Visual acuity and contrast sensitivity (Chapter 1)

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Abstract

This study is concerned with the relationship between anatomical and physiological characteristics of the early visual system in the tammar wallaby and some of its basic visual functions such as visual acuity, contrast sensitivity, spectral sensitivity and colour vision. The behavioural visual acuity was estimated in dual forced choice experiments to be about 4.8 c/deg. The contrast sensitivity, measured by evoked potentials, showed a peak at 0.15 c/deg, but a slightly lower associated acuity of 2.7 c/deg, as compared to the behavioural estimate. An immunocytochemical study of the retina of the tammar wallaby showed that it is clearly rod dominated, but also contains a low percentage of cone photoreceptors (3-5%), belonging to two different cone classes. The retina shows an interesting topography with both cone populations having very distinct distributions. The short wavelength sensitive cones represent about 5-30% of all cones, depending on the retinal location. Based on these distributions and in conjunction with the already known ganglion cell distribution, it is suggested that the retina of the tammar wallaby can be divided into three distinct regions with different functional specialisations: Spatial sensitivity (ventral retina), spatial acuity (central streak) and spectral sensitivity (dorsal retina). This argument is based on the relative numbers of ganglion cells and the two different cone populations. The spectral sensitivity of the wallaby's middle wavelength sensitive cones was measured electrophysiologically to have a peak at 540 nm, whereas the rods were found to peak at 500 nm. An independent microspectrophotometric study of the rod sensitivity confirmed this estimate. The spectral sensitivity of the short wavelength sensitive cones could not be determined electrophysiologically and was estimated from behavioural experiments to peak at about 420 nm. The same behavioural experiments showed the wallaby to have acute colour vision of a dichromatic nature. From the results of these studies, the tammar wallaby can be characterised as having a spatial acuity of about 5 c/deg with a peak in contrast sensitivity at 0.15 c/deg. It has acute dichromatic colour vision, based on a 420 nm and a 540 nm cone pigment, and its retina shows a differentiated topography with three distinct regions, each hypothesised to be specialised for a particular task.

Introduction

The studies presented here are concerned with the relationship between the fundamental visual capabilities of acuity, contrast sensitivity, spectral sensitivity and colour vision in the tammar wallaby and the relevant features of the anatomy and physiology of its eye.

The marsupials add an interesting comparative perspective to our understanding of the relationship between the environment and the visual system in mammals. Eutherian mammals and marsupials probably separated in evolutionary history at some point during the Cretaceous period (Lillegraven 1979). The Australian marsupials in particular, underwent a strong radiation during which they produced a variety of forms and adapted to many different environments. By comparing marsupials with each other and with eutherian mammals, one can infer what the visual system of their common ancestors may have been like and also the way in which different environments mould and shape sensory systems. The separation of traits that have evolved in response to a particular environment and traits that are based on a common ancestry is not a straightforward process, however. The marsupials, having separated from the eutherian mammals at such an early date in evolutionary history, and yet being subject to many of the same environmental pressures as their present day eutherian counterparts, are ideal subjects to explore and separate the factors that led to the traits we see today. Before we can achieve this goal, we first need to know more about marsupials, however.

In recent years, the tammar wallaby has proved itself a valuable mammalian model for neuronal development. A growing number of studies, including many investigating the visual system, have taken advantage of the marsupials' enormous potential for developmental research (Cassidy et al. 1994; Taylor and Guillery 1994; Harman et al. 1995; Hoffmann et al. 1995; Dunlop et al. 1996; Ding and Marotte 1997; Dunlop et al. 1997; Freeman et al. 1997; Ho 1997; Marotte et al. 1997). The marsupials' unique reproductive system allows easy access to the young at a very early developmental stage. Wallabies are born just 28 days after conception, long before most sensory and motor systems are fully developed. In the visual system for

example, the optic axons have not reached the brain at birth and it will take 140 more days before the eyes open (Wye-Dvorak 1984; Ding and Marotte 1997). Developmental studies and studies on mature systems often interact in a mutually beneficial way. Together, both fields can provide important insight into questions that are difficult to answer when studying just one field in isolation. For example, the functional emergence of cortical subsystems during development is important in understanding the hierarchical layering in the adult animal. Once the subsystems are fully developed, it is more difficult to isolate their functions. At the same time, developmental studies are greatly facilitated by and should be viewed in the context of the final functional system. The studies presented in this thesis, are intended to provide some information about the fundamental characteristics of the mature visual system of the tammar wallaby.

In order to understand the processing of visual information one first needs to know, what information is extracted by the retina from the environment and passed on to the brain. Visual acuity, contrast sensitivity, spectral sensitivity and colour vision, are some of the basic parameters in which one can describe the retinal input that is limiting cortical processing. There are of course others, such as temporal resolution or motion sensitivity, which are not covered here, that may or may not be limited by the retina.

The overall plan of this work has been to correlate anatomical and physiological measurements of the retina with behavioural measurements and to see to what extent they agree. In such basic detection or discrimination experiments, one would, in general, expect the animals to be able to make use of the retinal output up to its limits and would expect a good correlation between behavioural, physiological and anatomical methods.

The studies reported in this thesis, are presented in four chapters, followed by a brief overall summary. The chapters are organised as independent papers that have been published (Hemmi and Mark 1998) or submitted for publication. Between each chapter is a short linking section. A brief general introduction to each chapter follows.

Visual acuity and contrast sensitivity (Chapter 1)

Visual acuity, or more generally contrast sensitivity, is an important characteristic of an animal's eye design. It sets quite strict limits as to what information reaches the brain to be used for controlling various kinds of visual behaviours. Acuity is a very popular measure, because it can be expressed as a single number, which allows a large range of diverse animals and millions of bespectacled humans, for that matter, to be compared easily. Visual acuity, or more precisely resolution acuity, also seems to be related in a straightforward manner to the quality of the optics and the density of the neural sampling (see Hughes 1977 for a more detailed discussion). It is important, however, to keep in mind that when discussing resolution acuity, it is just one possible measurement of the quality of the eye. Some animals might find some forms of hyperacuities (Westheimer 1975) to be more important for their needs. Also, contrast sensitivity is not an isolated trait. The need of a fast moving animal to have a high temporal resolution will impact on its ability to achieve a high resolution acuity.

Even though there has been strong interest in the retinal topography of the ganglion cell distribution in a number of marsupials (Hughes 1975; Hokoç and Oswaldo-Cruz 1979; Tancred 1981; Silveira et al. 1982; Beazley and Dunlop 1983; Kolb and Wang 1985; Harman et al. 1986; Wong et al. 1986; Schmid et al. 1992; Dunlop et al. 1994; Arrese et al. 1998), only two studies have measured the visual acuity behaviourally (Harman et al. 1986; Arrese et al. 1998) and only one study has measured the contrast sensitivity using visual evoked cortical potentials (Silveira et al. 1982).

Therefore, the first study undertaken in the tammar wallaby set out to measure the behavioural visual acuity in a dual forced choice paradigm, using an experimental design that permitted the estimation of the influence of a range of factors on the animals' behaviour and therefore on the final acuity estimate. Due to the time-expensive nature of this type of experiment, visual evoked cortical potentials were used to complement the visual acuity measurement with an estimate of the complete contrast sensitivity function. The acuity can be predicted from the contrast sensitivity

and this estimate was compared with the behavioural acuity and the ganglion cell density.

Spectral sensitivity of the photoreceptors (Chapter 2)

The second chapter is concerned with the spectral sensitivity of the wallaby's photoreceptors. The spectral sensitivity of an animal's photoreceptors determines both, the amount of light the eye can absorb and the spectral range which is visible to the animal. In fish, for example, these constraints have led to a strong correlation between the spectral sensitivity of the rod photoreceptors and the animals' environment (e.g. Loew and Lythgoe 1978; Levine and MacNichol 1979; Lythgoe and Partridge 1991). In mammals, on the other hand, all species investigated seem to have a rod photoreceptor with a peak sensitivity around 500 nm (e.g. Lythgoe 1972; Bowmaker 1991). The spectral sensitivities of the cone photoreceptors has in recent years been measured in a number of mammals (see Jacobs 1993 for a review). To date, however, there has not been enough information about the animals' light environments in combination with their spectral sensitivities in order for a convincing correlation between the two to be attempted.

Despite the interesting evolutionary relationship between marsupials and eutherian mammals, only one study has tried to measure the spectral sensitivity of the photoreceptors in a marsupial, the Virginia opossum (*Didelphis virginiana*). This study provided evidence for the presence of only one cone pigment of about 560 nm peak sensitivity and has found no indication for a short wavelength sensitive cone class (Jacobs 1993).

In an attempt to measure the spectral sensitivity of the tammar wallaby's cones and rods, two different methods were used: Microspectrophotometry, which operates on the isolated cone outer segments and an electroretinogram based method, which measures the cone sensitivity in the intact eye, through the optics.

The retinal topography of the photoreceptors (Chapter 3)

Since evidence was found for only one cone photoreceptor in the tammar wallaby, just as has been the case in the opossum (Jacobs 1993), An immunocytochemical study was undertaken to determine whether this method could confirm the absence of any short wavelength sensitive cones (S-cones) in this retina. With the development of antibodies against specific visual pigments, it has become possible to identify cone classes belonging to different spectral sensitivities. This method has so far been very successful in predicting the presence of S-cones or middle to long wavelength sensitive cones (M/L-cones). The different antibodies seem to work across a range of species from chicken to humans, but cannot discriminate between M and L-cones in mammals (for summaries see: Szél and Röhlich 1989; Szél et al. 1996).

The second equally important reason for undertaking such a study has been that it allows a topographic analysis of the photoreceptor distributions. Such an analysis, as well as in primates, has now been undertaken in a range of mammals (Müller and Peichl 1989; Wikler and Rakic 1990; Petry et al. 1993; Juliusson et al. 1994; Röhlich et al. 1994; Szél et al. 1994; Ahnelt et al. 1995; Famiglietti and Sharpe 1995; Peichl 1997). It has become apparent that retinae are by no means as homogeneous as one might expect based on the primate retinae. Different animals sometimes show quite striking differences between the distributions of their cone classes and between cones and ganglion cells (Müller and Peichl 1989; Petry et al. 1993; Röhlich et al. 1994; Ahnelt et al. 1995).

Two different cone classes could be identified in the tammar retina and their topography mapped across the retina. The results are compared to published ganglion cell densities for the same species (Tancred 1981; Wong et al. 1986).

Behavioural colour vision (Chapter 4)

A second aspect connected to the spectral sensitivity of photoreceptors which was not mentioned above, is that of colour vision. If an animal has more than one photoreceptor it has the potential to compare the input of these photoreceptors and

therefore to see colour. This would literally add a whole new dimension to an animal's perception. Colour vision is a very powerful sense that enables its user to detect objects that do not provide a luminance contrast against the background or recognise objects despite that fact that they are cut into segments by strong luminance boundaries, such as those that may have been cast by the shadow of a tree.

Evidence is presented in the study of the retinal topography (chapter 3), for a low density S-cone population which is present throughout the retina. This is a strong indication for colour vision. However, there is only one way to show that this is a valid conclusion and that is through behavioural experiments. A carefully designed behavioural experiment can prove that an animal has colour vision, by showing that the animal can make discriminations between two lights based on their colour (hue), independent of their brightness. In addition, such an experiment will also enable the estimation of the spectral sensitivity of the photoreceptors involved.

A series of dual forced choice colour discrimination experiments was conducted in the tammar wallaby, using operant conditioning. The study showed that the wallaby does have dichromatic colour vision and characterised its quality in terms of the pigments involved. Based on the exact spectral sensitivity of the M-cones (chapter 2), it was finally possible to estimate the peak spectral sensitivity of the elusive S-cones.

Chapter 1

Abstract

The visual acuity of the tammar wallaby was estimated using a behavioural discrimination task. The wallabies were trained to discriminate a high contrast (80%)

Visual acuity, contrast sensitivity and retinal magnification in a Marsupial, the tammar wallaby (*Macropus eugenii*)

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and gratings of various spatial frequencies. The behavioural acuity was about 4.4 cycles/deg. The contrast sensitivity peaked at about 0.15 c/deg and declined towards higher spatial frequencies. The cut-off frequency of the contrast sensitivity function is slightly lower than the behavioural acuity at about 2.7 c/deg. The retinal magnification factor was estimated as approximately 1.6 times to be about 0.16 mm/deg. Based on the known ganglion cell density and the retinal magnification factor, an anatomical upper limit of visual acuity of about 6 c/deg can be calculated. The difference in estimates of visual acuity between the behavioural and anatomical methods on the one side and physiology on the other side are discussed.

Introduction

A range of macropodids have been investigated in regard to their ganglion cell distribution in the retina (Hughes 1975; Hobbs and Oswald-Cox 1979; Tansley 1981; Silveira et al. 1982; Koff and Wong 1983; Farmer et al. 1986; Wong et al.

Abstract

The visual acuity of the tammar wallaby was estimated using a behavioural discrimination task. The wallabies were trained to discriminate a high contrast (80%) square wave grating from a grey field of equal luminance ($1000-6000 \text{ cd/m}^2$). Visual evoked cortical potentials were used to measure the complete contrast sensitivity function. The stimulus was a sinusoidal phase reversal of a sinusoidally modulated grating of various spatial frequencies and contrasts with a mean luminance of 40 cd/m^2 . The behavioural acuity was estimated to be about 4.8 cycles/deg. The contrast sensitivity peaked at about 0.15 c/deg and declined towards both lower and higher spatial frequencies. The cut-off frequency of the contrast sensitivity function is slightly lower than the behaviourally measured acuity at about 2.7 c/deg. The retinal magnification factor was estimated anatomically from laser lesions to be about 0.16 mm/deg. Based on the known ganglion cell density and the retinal magnification factor, an anatomical upper limit to visual acuity of about 6 c/deg can be calculated. The differences in estimates of visual acuity between the behavioural and anatomical methods on the one side and physiology on the other side are discussed.

Wong et al. 1986).

The tammar wallaby is a small kangaroo like marsupial that weighs between 4-8 kg. On Kangaroo Island, South Australia, where this species occurs naturally, the wallabies spend the day in the scrub which they leave after dark to feed in the open grass plains during the night (Gray 1958). Animals in captivity show some activity throughout the day, but again are mostly active in the late afternoon and during the night (Blakers 1972, personal observations). Even though the tammar wallaby is a crepuscular animal, it is active during the day for a significant amount of time

Introduction

A range of marsupials have now been investigated in regard to their ganglion cell distribution in the retina (Hughes 1975; Hokoç and Oswaldo-Cruz 1979; Tancred 1981; Silveira et al. 1982; Kolb and Wang 1985; Harman et al. 1986; Wong et al. 1986; Schmid et al. 1992; Dunlop et al. 1994) and its development (Beazley and Dunlop 1983). The tammar wallaby in particular has received attention with studies ranging from the development of anatomy and functionality of the retino-cortical projections (e.g. Vidyasagar et al. 1992; Mark et al. 1993; Ding and Marotte 1997) electrophysiological studies of motion vision (e.g. Ibbotson et al. 1994) and the distribution sizes and classes of ganglion cells (Tancred 1981; Wong et al. 1986).

The present study is designed to provide behavioural and physiological information about the visual acuity of the mature visual system in the tammar wallaby. Visual acuity, or more general contrast sensitivity is a fundamental limiting characteristic of a visual system and needs to be taken into account when interpreting visual performances. The behavioural visual acuity was measured by means of a discrimination task and compared to a physiological measurement derived from the cut-off frequency of the contrast sensitivity function measured by Visual Evoked Cortical Potentials (VECP). We also measured the Retinal Magnification Factor (RMF), which allowed us to calculate a theoretical upper limit to visual acuity, based on estimates of the peak ganglion cell density from the literature (Tancred 1981; Wong et al. 1986).

The tammar wallaby is a small kangaroo like marsupial that weighs between 4-8 kg. On Kangaroo Island, South Australia, where this species occurs naturally, the wallabies spend the day in the scrub which they leave after dusk to feed in the open grass plains during the night (Inns 1980). Animals in captivity show some activity throughout the day, but again, are mostly active in the late afternoon and during the night (Blakers 1972, personal observations). Even though the tammar wallaby is a crepuscular animal, it is active during the day for a significant amount of time.

Materials and Methods

Behavioural experiments

Two mature male tammar wallabies were used in this study, both of which were bred at the Australian National University and raised in social groups in large outdoor paddocks. The two animals, Zauss and Flecke completed 1152 and 504 trials, respectively.

The experimental design used to measure the limit of visual acuity, was a two alternative forced choice paradigm. The tests were performed in the animal's normal home paddocks, where an area of 5 by 4 meters (figure 1-1) containing a two-arm maze was set aside. At the end of each arm, at 2.5 meters distance from the decision area, was a feeder that was marked either by a high contrast (86%) square wave grating pattern or a uniform grey field of equal space-averaged luminance. The targets were squares with 40 cm side length which had an angular size of 7.7° as seen from the decision area. The two feeders were separated by two opaque walls that were placed at an angle of 50° relative to each other. The entrance area was deliberately kept narrow in order to force the animal to approach the decision area straight on and slowly.

The animals were initially trained to distinguish a horizontal grating of 1.05 c/deg from a uniform grey field of equal average luminance. Correct decisions were rewarded by a few pellets of their usual supplement food, which was placed in the feeder just below the grating. The food was invisible from a distance greater than about 50 cm from the feeder. At the beginning of the training the animals were allowed to turn round after a wrong choice and inspect the second feeder (marked with the grating) and take the reward which was present there. Later in the training and during the tests, the animals had to leave the maze immediately after a wrong choice, after which the position of the grating (left or right feeder) was randomly changed before the animal was allowed to re-enter the maze and make a new choice. The wallabies learned to accept this procedure and usually left the maze of their own accord after a wrong choice.

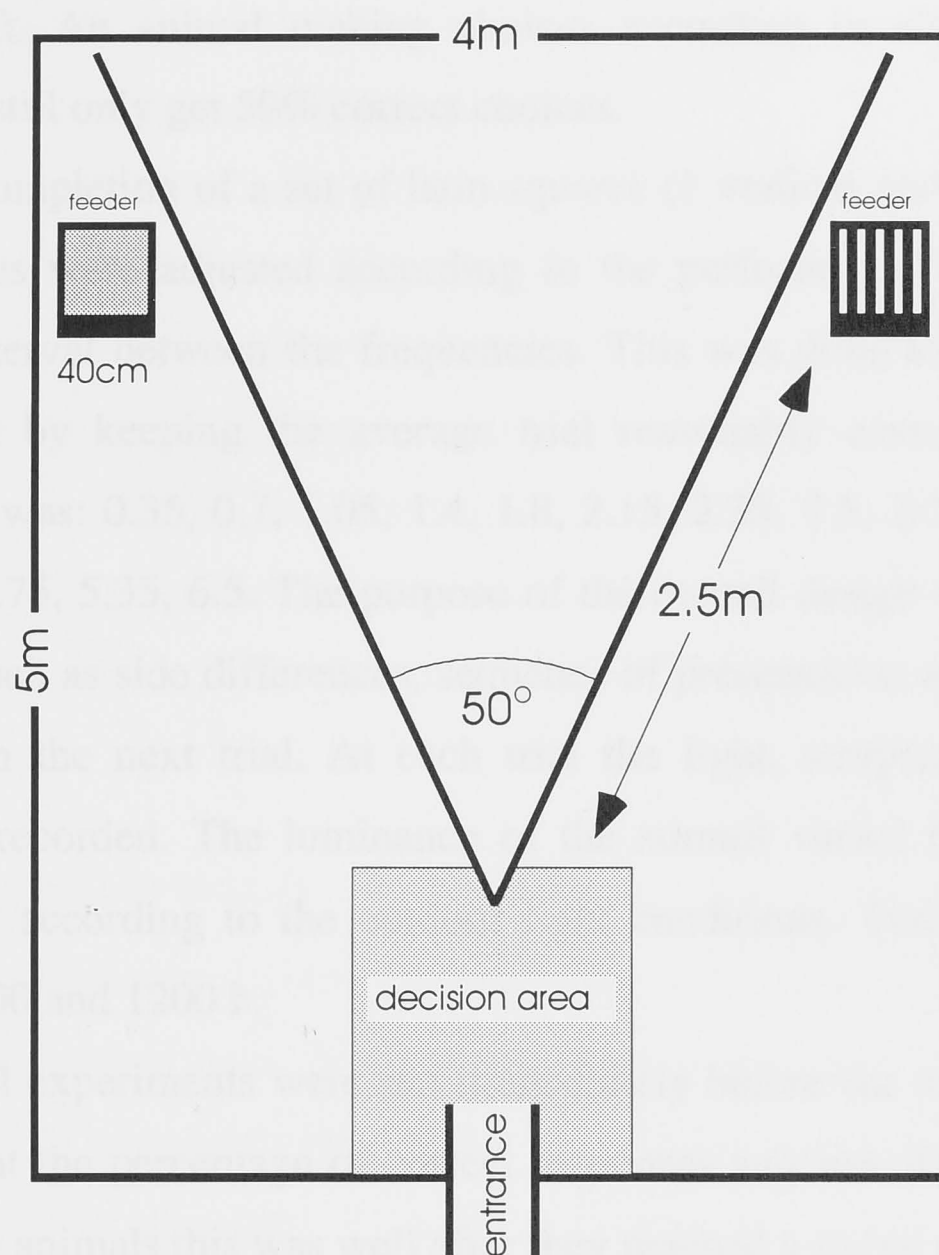


Figure 1-1: Plan of the experimental apparatus. The animal makes a choice by leaving the decision area towards either of the two feeders.

The spatial frequency of the square wave grating was varied between trials according to a 12 x 12 latin square design balanced for residual effects (Williams 1949). For any given latin square, twelve different frequencies were used. Each frequency was presented exactly once in each of the 12 blocks and every frequency was followed by every other frequency exactly once over the course of 12 blocks. Blocks of horizontal and vertical gratings were presented alternately. The position of the positive stimulus was varied randomly with the restriction that it was never associated with the same feeder more than three times in a row and never alternated between feeders more than three times in a row. This restriction was necessary in order to discourage the wallabies from falling back to spatial strategies such as win

stay or win shift. An animal making choices according to either of these two strategies would still only get 50% correct choices.

After the completion of a set of latin squares (1 vertical and 1 horizontal), the spatial frequencies were adjusted according to the performance of the animals by increasing the interval between the frequencies. This was done to keep the animals highly motivated by keeping the average trial reasonably easy. The total set of frequencies used was: 0.35, 0.7, 1.05, 1.4, 1.8, 2.15, 2.35, 2.5, 2.75, 2.85, 2.95, 3.2, 3.55, 3.9, 4.15, 4.75, 5.35, 6.5. The purpose of the overall design was to balance for random effects such as side differences, sequence of presentation and residual effects of frequencies on the next trial. At each trial the light, temperature and weather conditions were recorded. The luminance of the stimuli varied between 1100 and 6800 candelas/m² according to the outdoor light conditions. Testing was generally done between 0800 and 1200 h.

Two control experiments were run immediately before the main experiment in order to show that the percentage of correct responses was not affected by the food location. For both animals this was well after they reached a stable performance level. Trials alternated between a lower spatial frequency (1.05 c/deg) and a high spatial frequency (3.55 c/deg) and each was presented against a grey field of mean luminance. For the high spatial frequency trials the reward was placed under the positive stimulus (grating) only. For the low spatial frequency trials, the reward was placed under both, the positive and the negative stimulus (grey field). In the second control, the low spatial frequency had food under the positive feeder only, which could be compared to the case where the food was under both feeders.

Statistics

In order to measure the effects of the different experimental conditions on the animals responses, a Generalized Linear Model (GLM) of the following form was

fitted to the data: probit^1 (probability of success) = constant + effects due to variables and possible interactions between them

The random variation in the model was assumed to be binomial. An initial model $\text{probit}(p) = \text{Intercept} + \text{animal} + \text{block}$ was fixed to allow for differences between animals (2 level factor) and a change of response accuracy over the 4 sets of latin squares (blocks, 4 level factor). Other variables were then added to the model and their effects explored. Significant variables were included. All variables were tested for an interaction with frequency. Significance was judged according to the deviance differences between models (McCullagh and Nelder 1989).

Visual Evoked Cortical Potentials

Two adult tammar wallabies (male/female, 4.5 kg each) were used in the electrophysiological experiment. Both animals came from the same breeding colony as described above. The animals received an intramuscular injection of 0.46-0.65 mg Atropine Sulphate (Atrosine mitis, Parnell) 30 minutes prior to the experiment. They were first anaesthetised with an intravenous injection of 50 mg thiopentone sodium in a 5% solution (Pentothal, Boehringer) through a butterfly needle (23G) inserted into the lateral tail vein. Anaesthesia was continued using thiopentone sodium as required until the animal was put on a continuous iv. infusion of 3.6 mg/kg/hr of sodium pentobarbitone (Nembutal, Boehringer) in a compound sodium-lactate solution (Baxter) 5.6 ml/hr. The infusion also contained 7 mg/kg/hr of suxamethonium chloride (Scoline, DBL) to maintain paralysis of skeletal muscles. The animals were artificially ventilated with a 1:3 mixture of oxygen and nitrous oxide through a 4 mm Sheridan endotracheal canula. The animals' temperature was maintained at 37 degrees centigrade by a thermostatically controlled heating blanket. Before the animal was placed into the stereotaxic frame, the hair on its head was cut short, to allow the placement of subcutaneous electrodes. To assess the depth of the anaesthesia the electrocardiogram was continuously monitored. The CO₂ in the expired air was kept

1 The probit link was confined to between 0.5 and 11 i.e. $\text{probit}(p) = F(2p-1)$ where F = cumulative normal distribution.

between 4 and 5% throughout the experiment by adjusting the rate or stroke volume of the respirator. The animals were euthanased with an overdose of sodium pentobarbitone after completion of the experiment.

Visual evoked potentials were recorded using three silver/silver-chloride wires placed subcutaneously. The recording electrode was placed on top of V1, the reference electrode temporal on the animal's head behind the (closed) ipsilateral eye and the common earth was placed on the animals upper neck. The electrodes were connected to a two-stage preamplifier with a gain of 50,000, the output of which was fed into an A/D-converter. Cortical voltage responses were sampled at 1.6 kHz (exactly 16 times the frame rate). Stimulation and recording were both run from the same clock for exact synchronisation. Visual stimuli were generated on a computer controlled imaging display (Barco calibrator) driven by a Truevision ATVista graphics board at 100 Hz frame rate. The visual stimulus subtended an area of 30x30 degrees at a viewing distance of 0.5 m. The stimuli were a range of horizontal sinusoidal luminance gratings with different spatial frequencies and varying contrasts. The phase of these gratings was reversed at a temporal frequency of 4 Hz, according to a sinusoidal luminance modulation. The space averaged luminance was kept constant at 40.3 cd/m^2 . In the stereotaxic frame the animals' heads were held in such a way that the area of highest ganglion cell density, the visual streak, projected approximately to the horizon (Tancred 1981; Mark et al. 1993). The monitor was then placed 50 cm from the animal such that the centre of the grating pattern aligned with the horizon and then moved horizontally until a maximum response was recorded. For both animals, the final position was very close to being just in front of the animal, slightly offset towards the stimulated eye. This is consistent with expectations based on the location of the highest ganglion cell density which projects to directly in front of the animal (Tancred 1981; Wong et al. 1986; Mark et al. 1993). The opposite eye was kept closed by taping its lid with adhesive tape.

A 2.12 Diopter hard contact lens was placed onto the recording eye. The animal was then refracted by placing an additional lens in front of the eye. The strength of this lens was changed in steps of 0.5 D until the maximum response to a stimulus of

1.25 c/deg spatial frequency and a contrast of 0.5 was obtained. The final refraction was +3.12 D for both animals.

The contrast sensitivity was measured for eight spatial frequencies (0.07, 0.1, 0.13, 0.2, 0.4, 0.8, 1.6, 3.2 c/deg). The fast fourier transform was applied to the results and the second harmonic of the modulation frequency (in the complex plane) was compared to twenty neighbouring frequencies to determine whether it was significantly different from zero (James et al. 1995). For each spatial frequency-contrast combination recordings of 120 phase reversals were repeated at least 5 times and then until, a) the average of the second harmonic of the input frequency was significantly different from 0 ($p < 0.05$), or b) a maximum of 20 recordings were completed. If the second harmonic was found to be significantly different from 0, the contrast was lowered and the same routine was applied again. If it failed to become significant within the 20 trials, the contrast was increased and the same recording procedure repeated. The starting contrast for each frequency was close to one. Contrast sensitivity was determined as the inverse of the contrast for which at least two out of three such blocks showed a significant result and at the next lower contrast at most one out of three blocks showed a significant result. This method was adopted because in an initial investigation we did not find a clear linear or close to linear relationship between VECF amplitude and the logarithm of contrast (Jacobs et al. 1980, Silveira et al. 1982). Contrast was usually determined to within about one third of an octave.

The presentation of spatial frequencies was organised in such a way that all frequencies were presented in randomised order, each at its own current contrast. Then the contrast was individually adjusted for all frequencies as stated above and the next block of frequencies was tested. This assured that there were no large contrast (perceived contrast) changes between trials near cut-off contrast and that any changes in either signal or noise levels would affect all frequencies equally, therefore not distorting the shape of the contrast sensitivity function. The two highest frequencies were usually dropped about half way through the experiment in order to save recording time, because their contrast threshold was already clear at that point.

Estimation of the retinal magnification factor

In this experiment we used two female tammar wallabies (f407: 4.5 kg, f995: 6 kg) both had been under the same anaesthetic regime as described above. A "Hadron" Ruby-pulse laser was used to make full thickness laser lesions into the retina corresponding to known positions in visual space. The laser was projected through an ophthalmoscope that could be rotated in the horizontal and vertical direction around the centre of the eye. A rotation of the ophthalmoscope around this point resulted in a movement across the visible retina without loss of focus such as is consistent with a rotation around the nodal point of the eye. In one animal, f407, 7 lesions were made at regular intervals of 10° parallel to the horizon through the optical disk (OD), ranging from about 10° temporal of the OD to 50° nasal of the OD. At three points along this transect (50° , 30° , 0° nasal of the OD), an additional two lesions were made at 17.5° dorsal and 12° ventral of the horizontal transect. In the second animal, f995, three parallel, horizontal transects at 10° intervals were made. The middle transect crossed the optic disc. Each transect consisted of 9 to 11 lesions at 10° intervals. The animals were euthanased with the barbiturate Nembutal, administered intravenously. The eyes were immediately removed and the retina whole-mounted, with the choroid still attached. The retina of animal f407 was mounted fresh, whereas the retina of f995 was first fixed in 4% Paraformaldehyde for 2.5 h while in the eye cup. A camera lucida drawing was made of the laser lesions on the retina and the choroid. The retinal magnification factor was then calculated from the distances between these lesions by fitting a grid based on the corresponding positions in visual space to the retinal positions of the drawn lesions. A non-linear least-squares fitting algorithm was used, allowing for rotation and translation as well as for a horizontal and vertical magnification factor. Error estimates were derived by bootstrapping the result (Moulton and Zeger 1991). Fitting was straightforward and all results have been visually inspected.

Results

Behaviour

General aspects of training

It took some four months of daily training until the animals participated consistently and reliably in the experiment and until spatial response patterns, such as side preferences and alternation, had disappeared. Normally it was possible to complete two blocks (24 trials) per day per animal. After the training period, the animals seemed to try hard to make a correct choice and avoid errors. They would hop at great speed into the decision area, stop and slowly move their head to shift their gaze from one pattern to the other (often several times) therefore clearly facing each pattern before making a decision.

Responses to different spatial frequencies

The animals' responses were clearly frequency dependent. Figure 1-2 shows the combined responses from both animals averaged over all conditions. Numbers indicate the number of decisions at each spatial frequency. The performance of the wallabies declined with increasing spatial frequency of the grating, from almost 100% at low frequencies to about 50% at higher frequencies. Since the performance of the two animals was clearly similar and statistically not different ($df=1$, $d=1.03$, $p>0.1$, figure 1-3), we present the results from the two animals together. Based on the combined results from figure 1-2, we can estimate the visual acuity of the tammar wallaby to be about 4.4 c/deg at 60% correct choices.

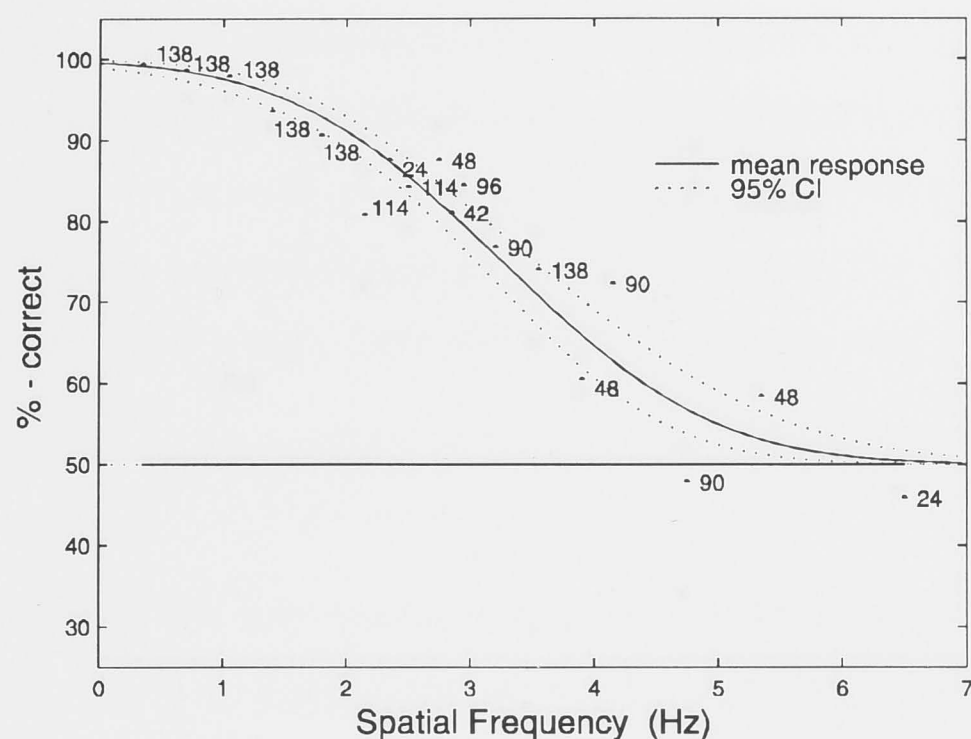


Figure 1-2: The percentage of correct choices plotted against the spatial frequency of the stimulus. The numbers refer to the number of decisions at each frequency. The lines correspond to the fitted mean function (see methods for the fitting routine) and the approximate 95 % confidence envelope for the mean. Depending on the cut-off criteria, the result is consistent with a visual acuity of about 4-5 cycles/degree.

Controls

Our control experiments showed that the percentage of correct responses was not affected by the food location. For the high frequency trials the animals chose randomly between feeders, even though only the feeder with the positive stimulus contained food (figure 1-4, column 1). When tested with a low spatial frequency, the percentage of correct choices was the same for both controls, regardless of whether the food was present in both feeders (figure 1-4, column 3), or only in the feeder with the positive stimulus (figure 1-4, column 2).

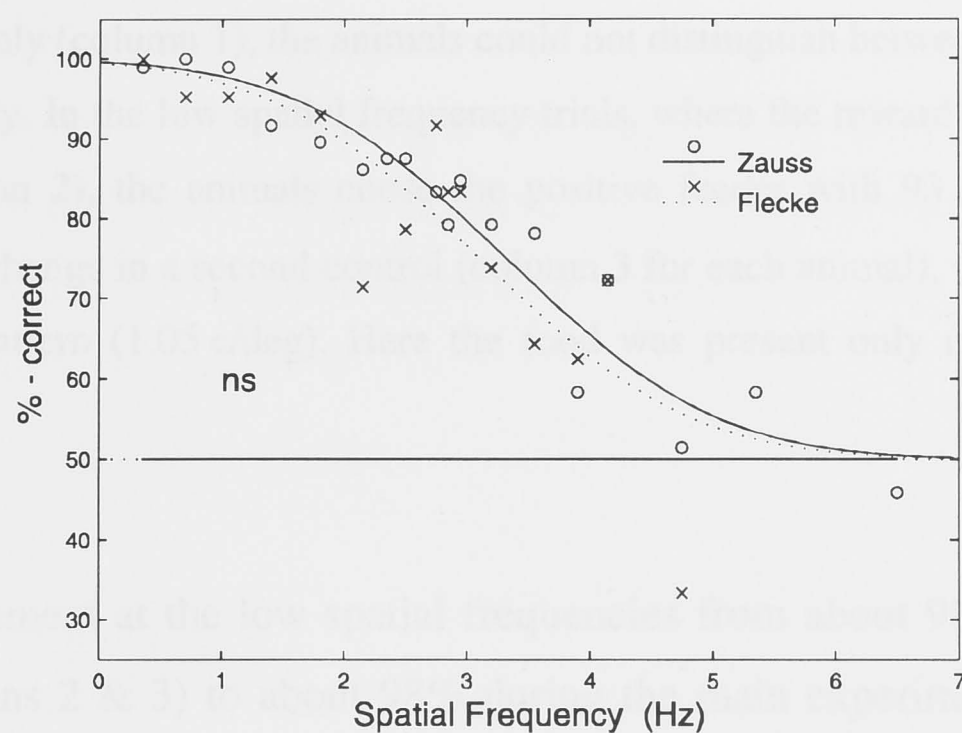


Figure 1-3: The percentage of correct responses for each of the two experimental animals plotted against the spatial frequency of the stimulus. The performance of both animals is very similar and their responses are statistically not different.

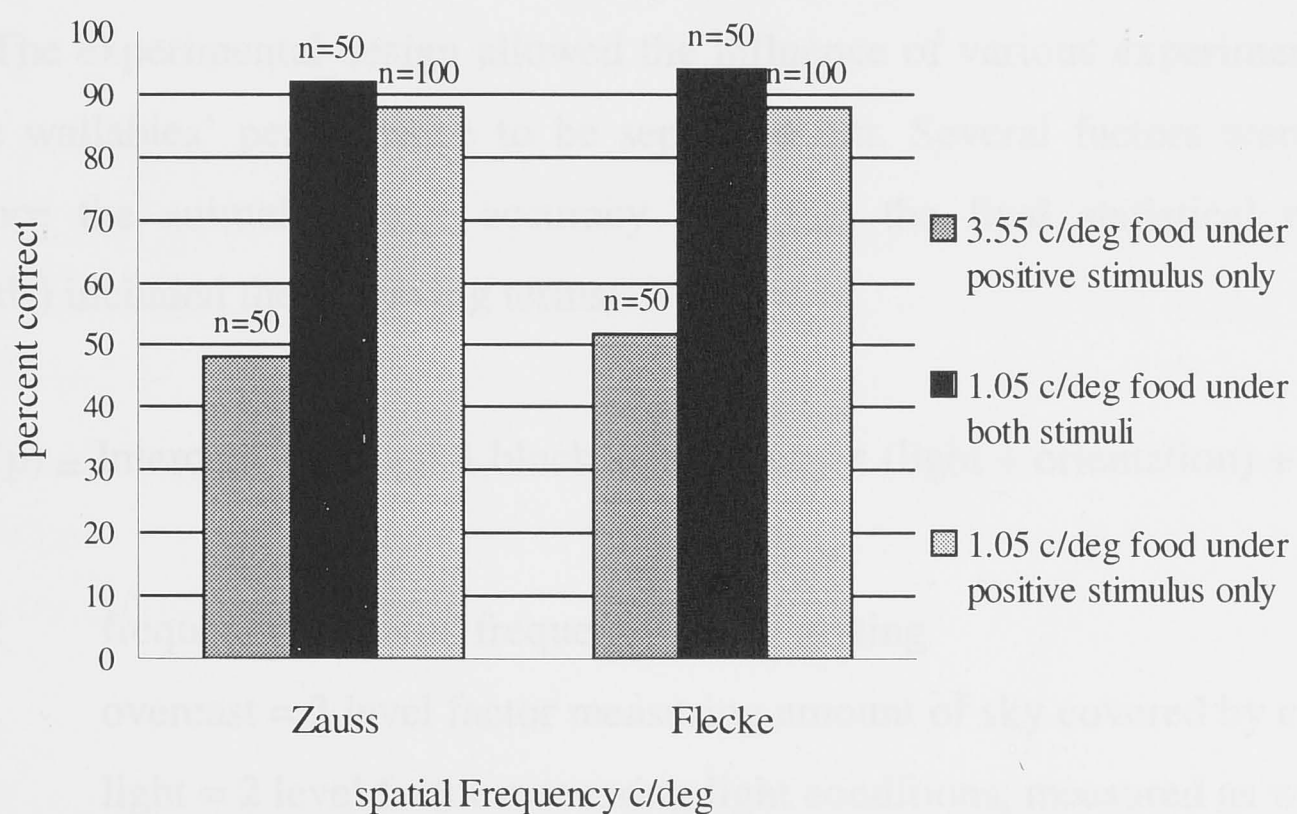


Figure 1-4: Two control experiments show that the percentage of correct responses is not affected by the food location. Trials alternated between a low spatial frequency (1.05 c/deg) and a high spatial frequency pattern (3.55 c/deg), each presented against a grey field of mean luminance. For the high spatial frequency trials the reward was placed under the positive

stimulus (grating) only (column 1), the animals could not distinguish between the two feeders and choose randomly. In the low spatial frequency trials, where the reward was placed under both feeders (column 2), the animals chose the positive feeder with 93 % accuracy. This percentage did not change in a second control (column 3 for each animal), with the same low spatial frequency pattern (1.05 c/deg). Here the food was present only under the positive feeder (88 %).

The improvement at the low spatial frequencies from about 90% correct trials (figure 1-4, columns 2 & 3) to about 98% during the main experiment (figure 1-2), did not seem to reflect an improvement in the wallabies performance over time. Rather, the fact that the wallabies were presented with a mixture of a wide variety of frequencies seemed to enhance both their motivation and accuracy. Note that the main experiment also has an in-built control in that, at high spatial frequencies the response is expected to approach 50%, as can be seen in figure 1-2.

Additional influences on performance

The experimental design allowed the influence of various experimental factors on the wallabies' performance to be separated out. Several factors were found to influence the animals choice accuracy such that the final statistical model (see methods) included the following terms:

$$\text{probit}(p) = \text{Intercept} + \text{animal} + \text{block} + \text{frequency} * (\text{light} + \text{orientation}) + \text{overcast}$$

where: frequency = spatial frequency of the grating
 overcast = 3 level factor measuring amount of sky covered by clouds
 light = 2 level factor measuring light conditions, measured as whether the animal cast a shadow onto the ground (sunny conditions) or not (shady conditions).
 orientation = 2 level factor measuring grating orientation.
 (see methods for a discussion of block and animal)

Figure 1-5 shows the effect of the different light conditions ($df=2$, $d=6.5$, $p<0.05$). The condition for a particular trial was judged to be sunny (which it was in the vast majority of the trials), when the animal cast a shadow on the ground. This turned out to be almost perfectly correlated with a mean luminance of the grating of more than 2200 cd/m^2 .

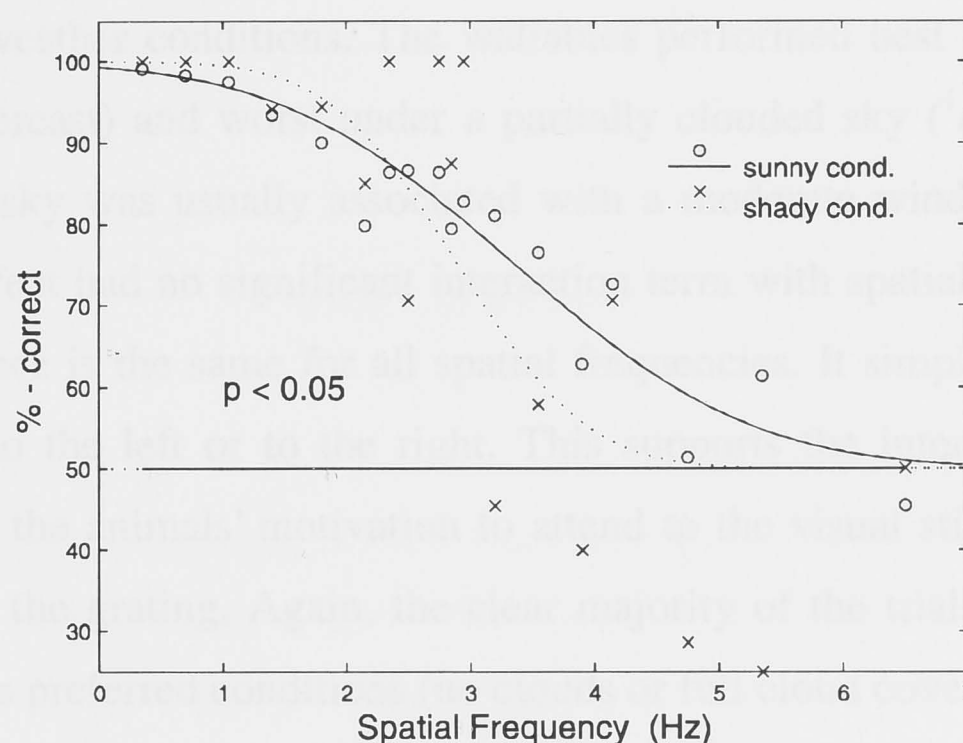


Figure 1-5: The percentage of correct responses under different light conditions (sun or shade) plotted against the spatial frequency of the stimulus. Under sunny light conditions (high mean intensity), the reduction in percentage of correct choices with spatial frequency is slower than for shady conditions (low light intensities, diffuse illumination). The experimental conditions did not allow us to determine whether this is due to differences of absolute light levels or to differences in light quality, such as how diffuse the light was under the two conditions.

It is not possible to distinguish with the data at hand whether the effect is due to the brightness of the targets or to a change in light quality. During shady conditions (lower light intensities), the light was much more diffuse than during sunny conditions (higher light intensities). The effect of this variable is frequency dependent which means its influence depends on the spatial frequency of the grating. Under high light intensities (sunny conditions), the decline in correct responses with increasing

spatial frequency was slower than for lower light conditions. It is important though to keep in mind, that even the lowest light intensity of the grating (1100 cd/m^2) is significantly higher than could normally be achieved under normal laboratory conditions. The high variance found in the results for shady conditions is due to few repetitions, especially at the higher frequencies.

Figure 1-6 shows the influence of the amount of cloud in the sky on performance ($df=2$, $d=7.6$, $p<0.05$). The amount of cloud is a good measure for the stability of the weather conditions. The wallabies performed best under a clear sky (less than $1/3$ overcast) and worst under a partially clouded sky ($1/3$ - $2/3$ overcast). A partially cloudy sky was usually associated with a moderate wind and a change in weather. This effect had no significant interaction term with spatial frequency which means its influence is the same for all spatial frequencies. It simply shifts the mean response curve to the left or to the right. This supports the interpretation that the weather affected the animals' motivation to attend to the visual stimulus rather than its ability to see the grating. Again, the clear majority of the trials were performed under the animals preferred conditions (no clouds or full cloud cover), which explains the higher variance found in the results for a partial cloud cover. The effect of the clouds is independent of the light conditions as discussed above and its effect persists after the data has been adjusted for different light conditions and vice versa.

Applying the criterion of 60% correct choices, the performance of the wallabies improves slightly from about 4.4 c/deg under average experimental conditions to about 4.8 c/deg under favourite conditions, i.e. sunny conditions and no clouds.

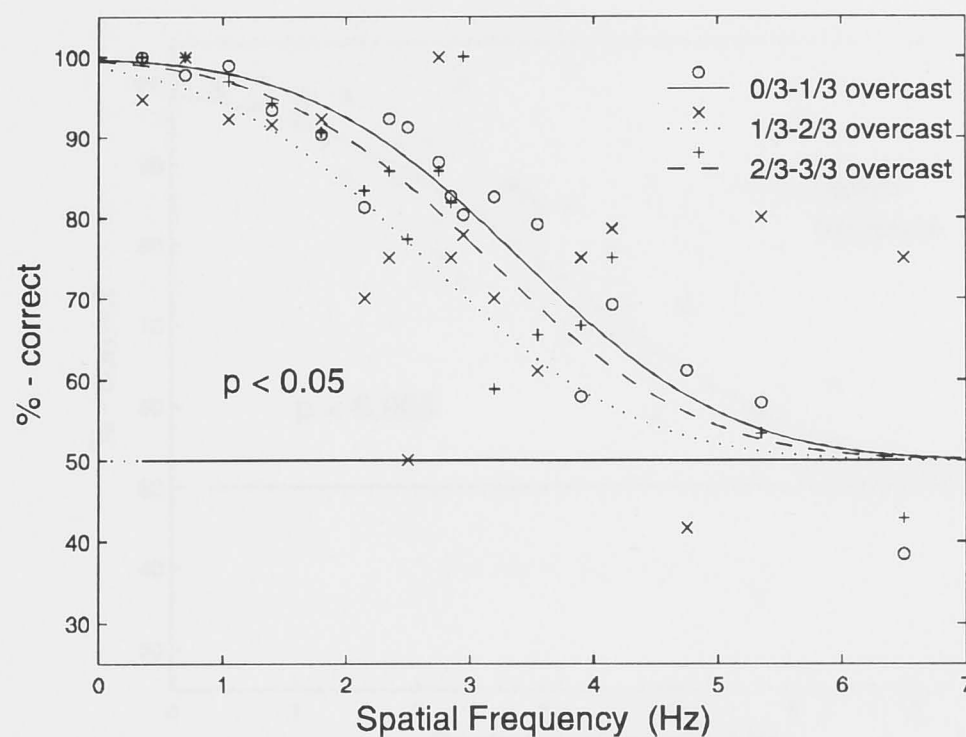


Figure 1-6: The percentage of correct responses under different weather conditions. The animals performed best under clear sky and worst, under a partially cloudy sky. This effect was found to be significant, even after adjusting for light conditions. The effect of the weather is the same for all frequencies, supporting the interpretation that the unstable weather affected the animals' motivation, thus, increasing the number of trials in which the animals ignored the visual cues completely. The graph illustrates the sensitivity and reliability of the technique used. Adverse factors influencing the animals performance could be quantitatively assessed and taken into account when interpreting the data to estimate the visual acuity.

The influence of grating orientation is again frequency dependent ($df=2$, $d=11.38$, $p<0.005$, figure 1-7). At lower frequencies the animals made clearly more errors with vertical than with horizontal gratings. The reverse seems to be true for higher frequencies. It was obvious throughout the experiment that the animals showed a slight hesitation to choose the lower vertical frequencies. They would often take a step towards the grating and then, before crossing the decision line, turn around and move a meter towards the grey field before turning around again. Unfortunately, the initial training was done with horizontal gratings for both animals and this effect may therefore be a training effect.

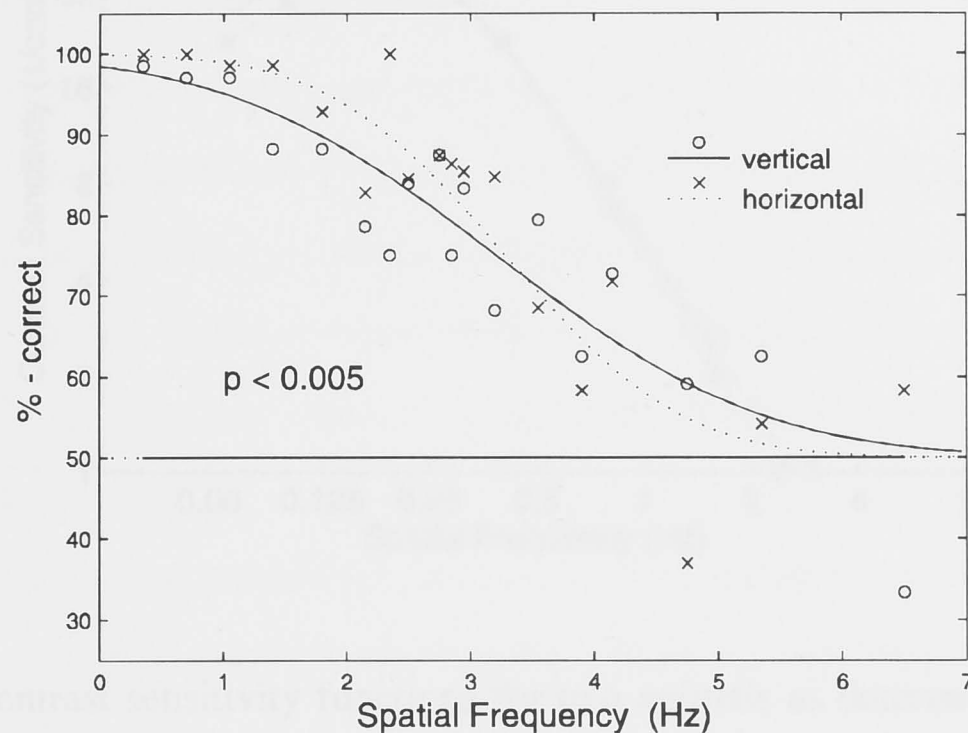


Figure 1-7: The percentage of correct responses for horizontal and vertical gratings plotted against the spatial frequency of the stimulus. At low frequencies the animals performed better for horizontal gratings, but at high frequencies this difference disappears or even reverses slightly. This effect is thought to be a training effect (see text for details).

Contrast sensitivity

The results from the VECP experiments are presented in figure 1-8 where the contrast sensitivity measurements for two animals have been plotted along with a thin plate spline function through the data points (Wahba 1990). The results obtained from the two animals are very similar. The contrast sensitivity shows a peak at around 0.15 c/deg and declines toward higher and lower spatial frequencies. The extrapolated visual acuity based on these curves for the two animals are 2.4 and 3.0 c/deg with a mean of 2.7 c/deg.

At the highest spatial frequency used in this experiment (3.2 c/deg), we could never obtain a significant response within the 20 repetitions even at full contrast. With further averaging (60-80 trials), however, a positive result was achieved.

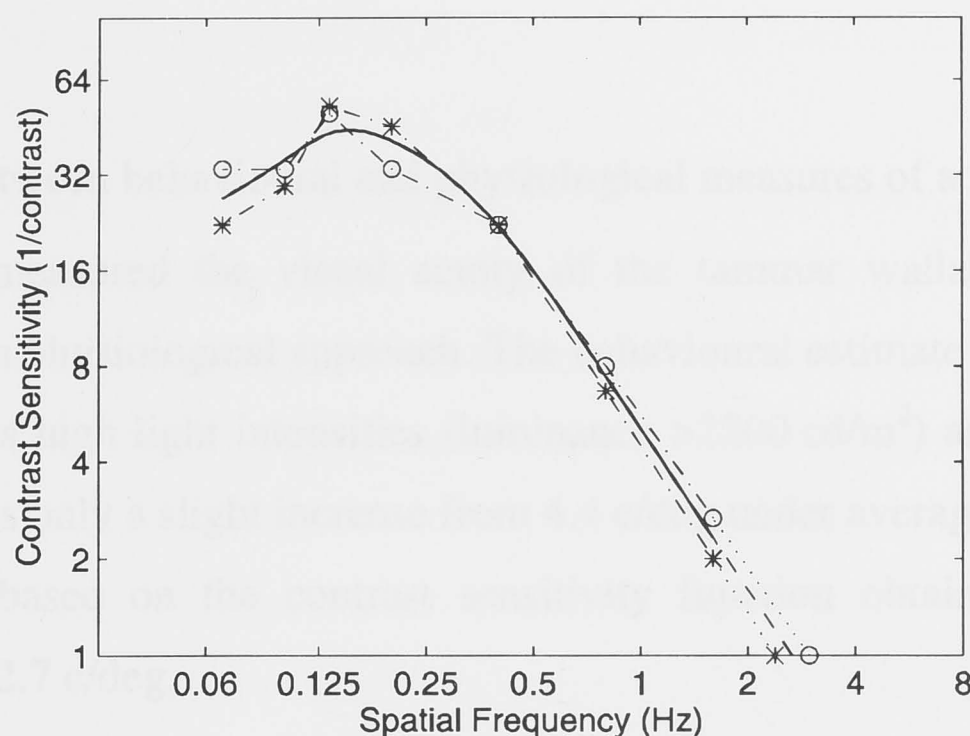


Figure 1-8: The contrast sensitivity functions for two animals as determined by the cortical VEP. The results are very similar with a peak in sensitivity around 0.15 c/deg. The highest spatial frequency from which significant results could be obtained under the experimental conditions was 1.6 c/deg (but see text for higher spatial frequencies). From this graph we can estimate the visual acuity to be about 2.7 c/deg (mean of both animals).

Retinal Magnification

For f407, the result of the retinal magnification factor is based on 9 clearly visible lesions that could be attributed to particular laser locations. The fitting routine gives a horizontal RMF of 0.162 ± 0.0035 mm/deg (mean \pm ste) and a vertical RMF of 0.157 ± 0.0063 mm/deg. For animal f995 28 lesions were clearly visible. The resulting horizontal RMF was 0.159 ± 0.001 mm/deg and the vertical RMF of 0.168 ± 0.0036 mm/deg. For animal f995 the vertical RMF is slightly higher than the horizontal RMF. For the purpose of calculating an anatomical upper limit to visual acuity, we used the mean of these measurements, 0.161 mm/deg. The ganglion cell distribution has been looked at in two different studies, both of which agree on a highest total ganglion cell density of about 5600 cell/mm² (Tancred 1981; Wong et al. 1986). Using the sampling theorem (Shannon and Weaver 1949, see 1977 for a discussion) we arrive at an anatomical upper limit for acuity of 6 c/deg.

Discussion

A comparison between behavioural and physiological measures of acuity

We have measured the visual acuity of the tammar wallaby using both a behavioural and a physiological approach. The behavioural estimate under favourable conditions, that is high light intensities (luminance $>2200 \text{ cd/m}^2$) and a clear sky, is 4.8 c/deg which is only a slight increase from 4.4 c/deg under average conditions. The acuity estimate based on the contrast sensitivity function obtained by VECF is slightly lower at 2.7 c/deg.

Before accepting the results of the behavioural experiments, it is important to be sure that the animals performed reliably. Working outdoors in the wallaby's normal home paddock could potentially have introduced a range of factors influencing the animals' accuracy. It had the distinct advantage, however, of keeping these shy animals calm and focused on the task. Besides, visual performance in the animals' natural setting is of behavioural significance. Even though the wallabies were rather difficult to train, once they had learned the task and were accustomed to the procedure, they performed very well, often taking seconds to make a decision, even when originally approaching the apparatus at considerable speed. The experimental design and the statistical treatment enabled us to measure the influence of a large range of factors on the animals choice behaviour. Only two factors were found to be significant, light and weather conditions and their influence on the acuity estimate was rather small (figure 1-5 & 6).

A partial explanation for the lower acuity estimates obtained with the physiological method might be found in the much lower light intensities used (40 cd/m^2 as opposed to $1100\text{-}6900 \text{ cd/m}^2$ for the behavioural experiments). The behavioural experiments did suggest that the wallabies performed better under brighter light conditions (figure 1-5), although it cannot be ruled out that the improvement was not due to a change in light quality (at lower light intensities, the light was also more diffuse, see results). In comparison, tree squirrels' visual acuity improved almost two fold from 2.2 to 3.9 c/deg when the luminance of the test gratings changed from 3.4 to 340 cd/m^2 (Jacobs et al. 1982). In contrast though, the

South American opossum shows no significant change in visual acuity with a two log unit change in light levels (Silveira et al. 1982).

A second reason for the discrepancy may be a methodological one. Campbell and Kulikowski (1972) showed that if the threshold contrast is determined by extrapolating the VECF recordings to the contrast which would produce zero voltage, the contrast sensitivity matches the behavioural sensitivity. The same was later found to be true in the cat (Campbell et al. 1973; Bisti and Maffaei 1974). In the present study contrast threshold was determined, without extrapolation, as the lowest contrast for which a response that was significantly different from zero could be recorded. This procedure leads to a decrease in the contrast sensitivity across all frequencies, and therefore of the acuity estimate, but it preserves the shape of the contrast sensitivity function. Extrapolation to zero voltage would shift the contrast sensitivity curve vertically upwards towards higher sensitivities and would therefore shift the predicted acuity cut-off to towards higher spatial frequencies. The fact that we were able to record significant responses to a 3.2 c/deg grating when we increased the number of presentations is consistent with this idea.

Anatomical upper limit

An anatomical upper estimate of visual acuity can be calculated from the ganglion cell density based on the sampling theorem (Shannon and Weaver 1949, see Hughes 1977 for a discussion). Thibos (1987) argued that, in the fovea of humans the quality of the optics threshold rather than the ganglion cell density sets the limit for the resolution (Campbell and Gubisch 1966), while resolution in the periphery is limited by the density of the beta or midget ganglion cell sub-population. It is unlikely, however, that the ganglion cell density exceeds the optical image quality in any part of the retina and it should therefore provide a valid upper limit to visual acuity (as opposed to hyperacuity, Westheimer 1975). The highest total ganglion cell density in the tammar wallaby is about 5600 cell/mm² (Tancred 1981; Wong et al. 1986). This value was arrived at by counting all classes of ganglion cells. The calculated estimate of visual acuity should therefore slightly overestimate the true acuity. The anatomical upper acuity limit calculated in this way, 6 c/deg, fits

reasonably well with the behavioural results (4.8 c/deg), but is a factor of 2 higher than the VECF results. The anatomical estimate is, of course, entirely dependent upon the actual proportion of ganglion cells involved in the respective spatial task. Silveira et al. (1982) using the extrapolation method, also found a twofold mismatch between the VECF-acuity and the anatomical estimate in the South American opossum and argued that only a fraction of the ganglion cells are processing spatial information. The close fit between the behavioural and anatomical acuity estimates in the tammar suggest, however, that the majority of ganglion cells are involved in the acuity task.

Comparison with other mammals

A comparison of contrast sensitivity in the tammar wallaby with that of other mammals can be seen in figure 1-9. Even with the threshold criteria used in this study, the highest sensitivity of the tammar is higher than that of most smaller mammals with the exception of the rabbit and the cat. On the other hand, the behavioural acuity of the tammar wallaby is slightly higher than that of the rabbit (2-3 c/deg, Van Hof 1966; Vaney 1980), but again lower than that of the cat (5-9 c/deg, e.g. Mitchell 1991).

In conclusion, the contrast sensitivity of the tammar wallaby has a standard band-pass shape with a peak sensitivity at about 0.15 c/deg. The visual acuity predicted by the contrast sensitivity function is slightly below the behavioural visual acuity which is an indication that this result slightly underestimates the true capabilities of the behaving animal. The behavioural visual acuity on the other hand is in good agreement with the retinal anatomy of this species (Tancred 1981; Wong et al. 1986). The spatial sensitivity seems quite limited, but when in danger the wallabies hop at an enormous speed through the scrub and may therefore have invested more into their temporal resolution. It would be interesting to look at temporal sensitivity to see whether one finds the same combination of low spatial and high temporal resolution in the tammar as has been found in tree shrews (Schafer 1969; Petry et al. 1984) and squirrels (Jacobs et al. 1980), which are both strongly diurnal groups.

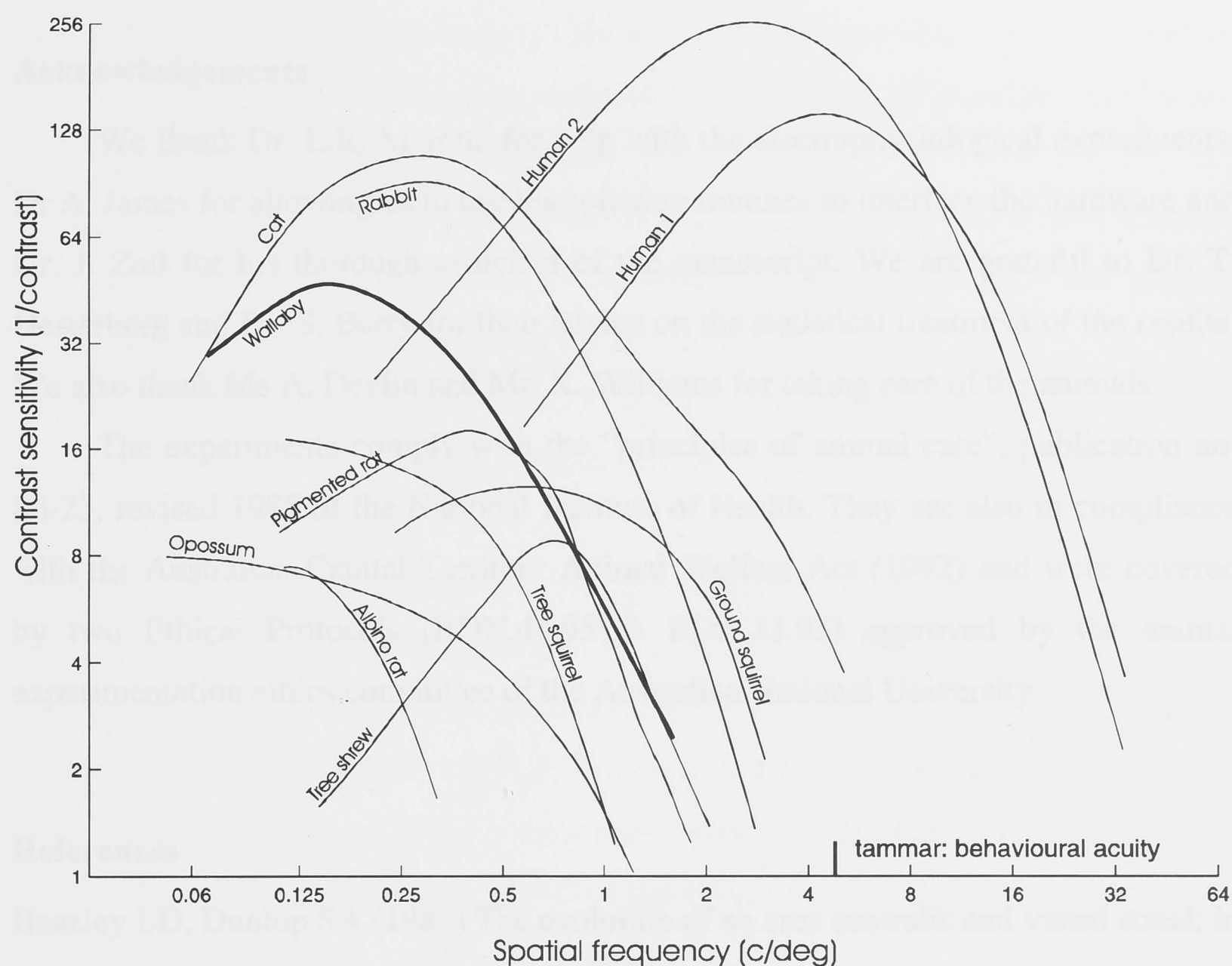


Figure 1-9: Comparison of the behaviourally or electrophysiologically determined spatial contrast sensitivity functions for a variety of mammalian species. Solid curves are thin plate splines (Wahba 1990) through the data points of the following published studies. Behaviourally determined CSF, Human: (1) DeValois et al. 1974, (2): Birch and Jacobs 1979; Cat: Bisti and Maffaei 1974; Tree squirrels: Jacobs et al. 1982; Rats: Birch and Jacobs 1979; Electrophysiologically determined CSF, Ground squirrel: Jacobs et al. 1980; South American opossum: Silveira et al. 1982; Tree shrew: Petry et al. 1984; Rabbit: Pak 1984; Tammar wallaby: present study.

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The experiments comply with the "principles of animal care", publication no. 86-23, revised 1985 of the National Institute of Health. They are also in compliance with the Australian Capital Territory Animal Welfare Act (1992) and were covered by two Ethical Protocols (RDN.44.95 & RDN.31.93) approved by the animal experimentation ethics committee of the Australian National University.

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From spatial to spectral sensitivity

The first chapter reported experiments showing that the visual acuity of the tammar wallaby is comparable to that of a range of other mammals of similar size. The contrast sensitivity predicted a slightly lower visual acuity than actually observed in the behavioural experiment, for reasons discussed. When the contrast sensitivity function is shifted vertically to match the behavioural estimate, its highest sensitivity is comparable to that of the cat and the rabbit, but is inferior to humans.

As mentioned previously, acuity or contrast sensitivity is just one measure of an animal's visual capabilities. Another important, if not even more fundamental aspect of early vision, is the spectral sensitivity of the photoreceptors. Photoreceptors are the interface between the light environment and the visual system. Whatever information is lost at this point cannot be reconstructed afterwards, however good an animal's acuity or visual processing may be. A successful eye design does not necessarily have to capture the entire spectrum, however. It can limit itself to the region where the biologically relevant information is contained. An instructive example for a specialised spectral sensitivity profile is provided by some rodents, the common house mouse (*Mus musculus*) for instance. Different from the typical mammalian pattern of spectral sensitivities, the sensitivity of their short wavelength sensitive cone (S-cone) is shifted to have a maximum sensitivity in the UV range (Jacobs and Neitz 1991). Even more surprising, the distributions of their two cone classes only overlap in the dorsal retina (Szél et al. 1992). Since colour vision depends on the input of at least two spatially co-localised cone populations, these animals are unlikely to have colour vision in large parts of their visual fields. This has not yet been shown behaviourally, however. The spectral sensitivities of their cones may therefore be optimised to luminance contrast rather than colour. To measure the relationship between an animal's biologically relevant light environment and the spectral sensitivity of its photoreceptors is not a trivial task (e.g. Endler and Théry 1996). Before it can be attempted, one needs to know the spectral sensitivity of all its photoreceptors. The following chapter describes the experiments designed to provide the spectral sensitivity data for the tammar wallaby.

Chapter 2

Abstract

Microspectrophotometric measurements were made on the photoreceptors of the tammar wallaby showing that they have a standard spectral sensitivity with a peak spectral sensitivity at about 300 nm.

Spectral sensitivity of photoreceptors in an Australian marsupial, the tammar wallaby (*Macropus eugenii*)

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electroretinogram measurements also confirmed the peak spectral sensitivity of the rods to be at 300 nm. We give reasons why the electroretinogram measurements might have failed to identify the presence of a short wavelength sensitive cone.

Introduction

In recent years our knowledge about primate vision has greatly increased. A range of species, in addition to primates, have now been shown to possess colour vision and the spectral sensitivity of a substantial number of mammals

Abstract

Microspectrophotometric measurements were made on the rod photoreceptors of the tammar wallaby showing that they have a standard mammalian rhodopsin with a peak sensitivity at 501 nm. An electroretinogram based study into the spectral sensitivity of the cone photoreceptors provided clear evidence for one cone pigment with a peak spectral sensitivity at about 539 nm. Despite a considerable effort, we failed to find evidence for a short wavelength sensitive cone class. The electroretinogram measurements also confirmed the peak spectral sensitivity of the rods to be at 500 nm. We give reasons why the electroretinogram measurements might have failed to identify the presence of a short wavelength sensitive cone class.

contrast, immunocytochemical studies in several species, including the grey short-tailed opossum (*Monodelphis domestica*, Water and Rausa 1979) and the South American opossum (*Didelphis marsupialis*, Erving *et al.* 1983) which belongs to the same genus as the Virginia opossum, suggest that the retina of these species do indeed contain S-cones, although it is doubtful. The differences between these results have not been resolved to date.

In the following study, we set out to measure the spectral properties of the tammar wallaby in order to find out whether its retina contains any short wavelength classes, and therefore provide the potential for behavioural colour vision. The tammar wallaby is a kangaroo like Australian marsupial (order *Dipodomys*) group that went through an extensive collection and process in 1978-79. The species including the kangaroo/wallabies, the Australian possum, and the wallaby. The adult tammar weighs between 4-8 kg and inhabits the scrubland of the south-east of Kangaroo Island, South Australia, where there is a large population. It has been seen to forage in the open grassy fields, but it is also known to be hidden in the scrub during daylight hours. It is a nocturnal animal, but shows some activity throughout the day, but appears to be most active at the time of sunset.

Introduction

In recent years our knowledge about colour vision in mammals has greatly increased. A range of species, in addition to primates, have now been shown to possess colour vision and the spectral sensitivity of a substantial number of mammals has been described in detail (reviewed by: Jacobs 1981; 1993). As Jacobs points out, however, information about marsupial colour vision is almost non-existent. The only exception is the Virginia opossum (*Didelphis virginiana*), an early American member of the marsupialia. The opossum was shown by Friedman (1967) to be able to distinguish between several colours and grey over a range of intensity ratios, thus presenting clear evidence for behavioural colour vision. For this same species, Jacobs and his co-workers later measured the spectral sensitivity of the cone photoreceptors with their flicker photometric procedure, an ERG based method. They found one cone class with a peak spectral sensitivity at about 560 nm, but failed to find any evidence for a short wavelength sensitive cone class (S-cones) (Jacobs 1993). In contrast, immunocytochemical studies in two other opossum species, the grey short-tailed opossum (*Monodelphis domestica*; Wikler and Rakic 1990) and the South American opossum (*Didelphis marsupialis aurita*; Ahnelt et al. 1995) which belongs to the same genus as the Virginia opossum, suggest that the retinae of these species do indeed contain S-cones, although at low density. The differences between these results have not been resolved to date.

In the following study, we set out to measure the spectral sensitivity of the tammar wallaby in order to find out whether its retina contains two different cone classes, and therefore provides the potential for behavioural colour vision. The tammar wallaby is a kangaroo like Australian marsupial from the *Diprotodonta*, a group that went through an extensive radiation and produced a range of diverse species including the kangaroos/wallabies, the Australian possum and the koala. The adult tammar weighs between 4-8 kg and inhabits the southern parts of Australia. On Kangaroo Island, South Australia, where there is a large wild population, the tammar has been seen to forage in the open grass plains at night, but is also active while hidden in the scrub during daylight hours (Inns 1980). Animals in captivity show some activity throughout the day, but again, are mostly active in the late afternoon

and during the night (Blakers 1972, personal observations). Thus, even though the tammar is often characterised as a nocturnal or crepuscular species, it is active for a significant amount of time during the day.

Materials and Methods

Microspectrophotometry (MSP) of rod photoreceptors

Tissue preparation

Retinae were obtained from six animals euthanased by cervical displacement for unrelated experiments. The animals had come from a breeding colony and were kept in social groups in outdoor paddocks. The eyes were removed in the dark within 2-3 minutes after death and put on ice for 2-10 h before the actual measurements took place. For the preparation, the eyes were transferred into Dulbecco "A" phosphate-buffered saline (PBS; Oxoid Ltd., UK), pH 7.3. The following preparation was done with the help of an infrared image converter (FJW, USA) under far-red light produced by a long pass wratten filter (Kodak 87c) in front of a halogen torch. Small pieces of retina (ca. 1 mm²) were placed on a 50x25 mm microscope coverslip. The excess PBS was removed and replaced with PBS containing 10% Dextran (250RMM; Simga, UK) in order to increase the viscosity of the preparation. The tissue was then pulled apart with a needle and a scalpel to separate the photoreceptors, mounted under a coverslip, the edges of which were sealed with paraffin wax.

The microspectrophotometer (MSP)

The single beam microspectrophotometer has been described in detail by Partridge et al. (1992). The light is produced by a 12 V, 100 W quartz-tungsten halogen bulb and is imaged on a holographic grating monochromator (Jobin Yvon, H-1061, France). The light is then projected onto the specimen plane in the shape of a rotatable, variably sized rectangular aperture. The dimensions of this aperture are diffraction limited. The specimen and the measuring beam are then imaged through a 100 x oil immersion objective (Zeiss Neofluar) on to a photomultiplier (Hamamatsu R2928, Japan) or can be viewed through a video-camera/monitor (Insight Vision

Systems, UK, 75 series). The specimen is illuminated by a 6 V, 15 W light source through a long pass (50% cut-off at 850 nm) wratten filter (Kodak, 87c). The output of the photomultiplier is converted to a digital signal and logged by a computer. The software to control the MSP and the logging of the data was written by Dr. Julian C. Partridge.

Spectral absorbance measurements

To take a measurement, the measuring beam was first set to a wavelength of 750 nm and focused in a cell free area near an outer segment of a photoreceptor to record a baseline scan. The outer segment was then moved into the light beam and a second scan was completed. Both measurements consisted of a sweep from long (750 nm) to short wavelengths (390 nm) and back again, measuring transmitted light intensity in 2 nm steps. Absorbance at even wavelengths was measured during the sweep from long to short wavelength, and during the sweep from short to long wavelength, odd wavelengths were measured. The resulting sampling interval was therefore 1 nm.

Data analysis

Individual recordings were first digitally filtered with a zero phase-shift filter and then a retinal (A_1) based nomogram (Stavenga et al. 1993) was fitted to the data. The filtering process removed high frequency noise from the recordings, but did not distort the shape of the signal: When a nomogram was filtered with the same digital filter, the deviations between the filtered and unfiltered version was less than 0.05% of peak sensitivity. A non-linear least squares fitting routine (Levenberg-Marquardt) was used to fit the nomogram to the data. Only measurements between 460 and 750 nm were used for the fit. The best recordings were then selected by the criteria that the sum of the squared residuals in the normalised recordings be less than 0.5 in the range from 40 nm to the short wavelength side of the peak to 120 nm to long wavelength side of the peak. This selection is purely based on the amount of noise in the data relative to the shape of a nomogram and not on the location of the peak sensitivity.

Electroretinogram (ERG) measurements

Four tammar wallabies (3 males, 1 female) weighing between 4.5 and 6 kg were used in this study. The animals were from a different colony to that which supplied animals for the microspectrophotometry. They were also purpose bred for laboratory use and kept in small social groups in outdoor paddocks.

The animals received an intramuscular injection of 0.46-0.65 mg atropine sulphate (Atrosine mitis, Parnell) 30 minutes prior to the experiment. They were first anaesthetised with an intravenous injection of 50 mg Thiopentone Sodium in a 5% solution (Pentothal, Boehringer) through a butterfly needle (23G) inserted into the lateral tail vein. Anaesthesia was continued using Thiopentone Sodium as required until just prior to beginning recording, when the animal was put on a continuous intravenous infusion of 3.6 mg/kg/hr of sodium pentobarbitone (Nembutal, Boehringer) in a compound sodium-lactate solution (Baxter) at 5.6 ml/hr. The infusion also contained 7 mg/kg/hr of suxamethonium chloride (Scoline, DBL) to maintain paralysis of skeletal muscles. The animals were artificially ventilated with a 1:3 mixture of oxygen and nitrous oxide through a 4 mm Sheridan endotracheal canula. The animals' temperature was maintained at 37°C by a thermostatically controlled heating blanket. Before the animal was placed into the stereotaxic frame, the hair on its head was cut short, to allow the placement of subcutaneous electrodes. The electrocardiogram was continuously monitored. The CO₂ in the expired air was kept between 4 and 5% throughout the experiment by adjusting respirator rate or tidal volume. The animals were euthanased with an overdose of sodium pentobarbitone directly after completion of the experiment.

Electroretinograms were differentially recorded using three silver/silver-chloride wires. The recording electrode was placed 1 mm inside the vitreous through a small incision in the cornea. The other two electrodes were placed subcutaneously, the reference electrode just temporal of the recording eye and the common earth on the animal's cheek about equidistant to the other two electrodes. All electrodes were connected to a two stage preamplifier with a gain of 50,000, the output of which was fed into an A/D-converter. Visual stimuli were generated on a computer controlled

imaging projector (Barco, DATA800) driven by a Truevision ATVista graphics board at 80 Hz frame rate. Retinal voltage responses were sampled at 2.56 kHz, exactly 32 times the frame rate. Stimulation and recording were both run from the same clock for exact synchronisation.

In the stereotaxic frame, the animal's head was held in such a way that the area of highest ganglion cell density, the visual streak, projected approximately to the horizon (Tancred 1981; Wong et al. 1986; Mark et al. 1993). A tangential screen was placed 0.5 m from the animal such that the centre of the grating pattern aligned with the horizon. The horizontal placement of the viewing screen did not change the response amplitude by a large amount and we therefore chose a final position of about 30 degrees offset from the vertical meridian (directly in front of the animal) which gave good responses for all animals. The opposite eye was kept closed by taping its lid with adhesive tape.

The design employed to measure the spectral sensitivity of the wallabies' photoreceptors is a variation of the flicker photometric procedure introduced by Neitz and Jacobs (1984). Rather than measuring the response amplitude to monochromatic light stimuli, however, we measured the effectiveness of monochromatic lights in reducing the perceived contrast of a flickering broadband light stimulus. Figure 2-1 shows a bird's eye view of the experimental arrangement. The animal (top of figure) is looking at a tangential screen (middle). The stimuli were projected onto the screen from the opposite side (bottom). We measured the response amplitude of the wallabies eye to a sinusoidal contrast modulation of a single, white or broadband coloured, square check, presented on a grey background with equal time-averaged luminance. The check subtended an area of 30x30 deg at a viewing distance of 0.5 m and its contrast, in respect to the background, was sinusoidally reversed at either 20 or 40 Hz.

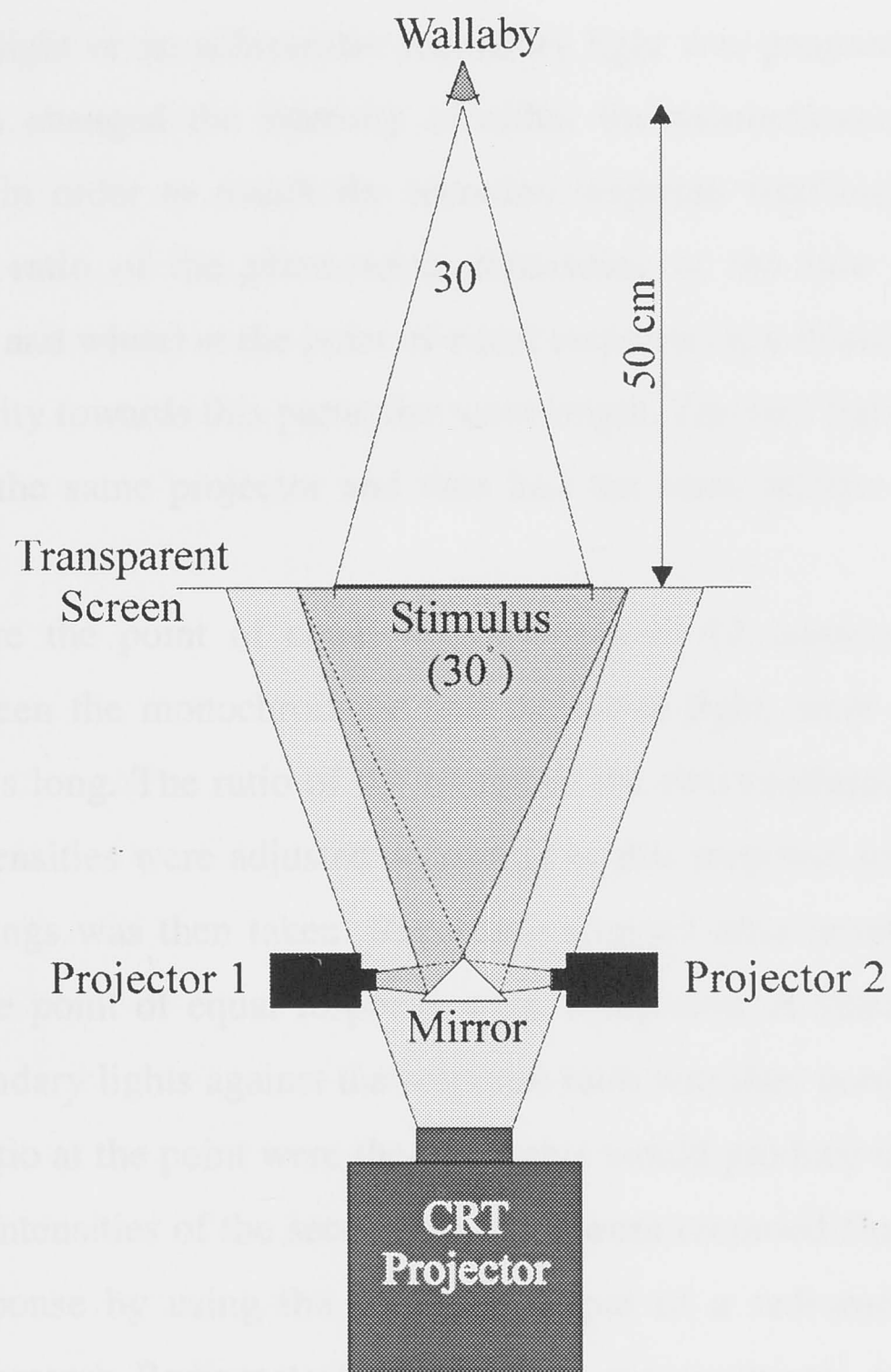


Figure 2-1: A bird eye's view of the experimental apparatus. The animal (at the top) is viewing a tangent screen (centre). A CRT-projector and two slide projectors (bottom) were used to project the stimulus onto the same screen from the other side. The three different light sources allowed a variety of light conditions to be produced.

By projecting secondary, unmodulated lights onto the same screen (figure 2-1, projector 1), we were able to reduce the contrast of the modulated check and thereby the amplitude of the animal's response. The secondary light was either an achromatic, white light or a monochromatic light with a half energy width of about 10-20 nm. In order to measure the spectral sensitivity of the eye for a given wavelength, we recorded the responses of the eye to the modulated check in the case where either a

monochromatic light or an achromatic secondary light was projected onto the same screen. We then changed the intensity of either the monochromatic or the white secondary light in order to match the recorded response amplitude under the two conditions. The ratio of the photometric intensities of the two secondary lights (monochromatic and white) at the point of equal response, is a relative measure of the animal's sensitivity towards this particular wavelength. The two lights were projected in sequence by the same projector and thus had the same relative spatial intensity distribution.

To measure the point of equal response, 10 to 12 consecutive recordings, alternating between the monochromatic and the white light, were completed. Each recording was 2 s long. The ratio of the means of the two conditions was calculated and the light intensities were adjusted according to this response ratio. A new set of 10 to 12 recordings was then taken. Recording stopped after several recordings on either side of the point of equal response were completed. A linear fit through the ratio of the secondary lights against the response ratio was then used to determine the light intensity ratio at the point where the two lights would produce the same response amplitude. The intensities of the secondary lights were recorded simultaneously with the animal's response by using the analogue output of a radiometer (International light: IL 700 Research Radiometer). Radiometric measurements were converted to photometric units for the sensitivity calculations. This design does not make any assumptions about the relationship between response amplitude and stimulus contrast apart from assuming the relationship to be monotonic.

The high contrast modulated check-stimulus and its time modulation was produced by a CRT-projector (Barco, DATA800) at about 80 cd/m^2 mean luminance. The secondary lights were produced by two slide projectors with 250 W halogen light bulbs (Philips).

Different cone classes were isolated by following the general protocol outlined in Jacobs et al. (1985). In order to measure the wallabies' middle to long wavelength cones (M-cones) a white stimulating check was used, the contrast of which was reversed at 40 Hz. The maximum contrast without a superimposed secondary light,

was about 80%. The mean luminance of the final stimulus (including secondary lights) varied between 80 and 200 cd/m^2 .

We attempted to measure the short wavelength sensitive cones (S-cones) by changing the recording procedures to a slower stimulation rate, concurrent intense long wavelength adaptation and a blue-dominated stimulation light (Crognale et al. 1991). This was done by modulating only the blue phosphor of the projection monitor and keeping the green phosphor at mean luminance and the red phosphor at its highest luminance at all times. Thus, we had a blue stimulating light on a yellow background. In addition, we used either a 590 nm band pass adaptation light (12 nm half energy band width) with an intensity of 104 cd/m^2 (animal f4301), or a 600 nm long pass adaptation light (Kodak cut-off filter no. 25 with 50% transmission at 600 nm) with an intensity of 690 cd/m^2 (animal m151096). The contrast of the blue check was reversed at 20 Hz. The contrast as seen by a pigment with peak sensitivity at 540 nm would have been reduced by a factor of 9 to about 9% (animal f4301) or by a factor of 22 to about 3.6% for animal m151096. For a short wavelength sensitive pigment at 440 nm on the other hand, the contrast would only have dropped by about 12% to a final contrast of 70% (both cases). All of these contrast measurements do not incorporate the secondary lights, which would further reduce the contrast seen by the photoreceptors.

The secondary lights varied between 10 and 100 cd/m^2 depending on wavelength. Due to the use of halogen light bulbs, there was much more light available for the longer wavelength, which made these measurements more reliable. Fits to the data were therefore confined to wavelength between 470 and 610 nm.

Results

Microspectrophotometry

The MSP measurements were restricted to rod measurements because it was found that the cones were so tightly associated with the pigment epithelium in the unfixed retina that removal of the pigment epithelium also removed the cone outer segments from the preparation. We therefore did not record from cone cells. Fourteen

recordings satisfied the selection criteria based on the noise in the recording as outlined in the methods section. Figure 2-2 shows the 14 selected recordings (dotted lines). The mean peak location is 501.4 ± 0.7 nm (mean \pm se). The solid curve shows a nomogram with peak location at 501.4 nm. The maximum deviation between the recordings and the fitted nomograms in the region used to select the recordings is less than 16% of the peak sensitivity.

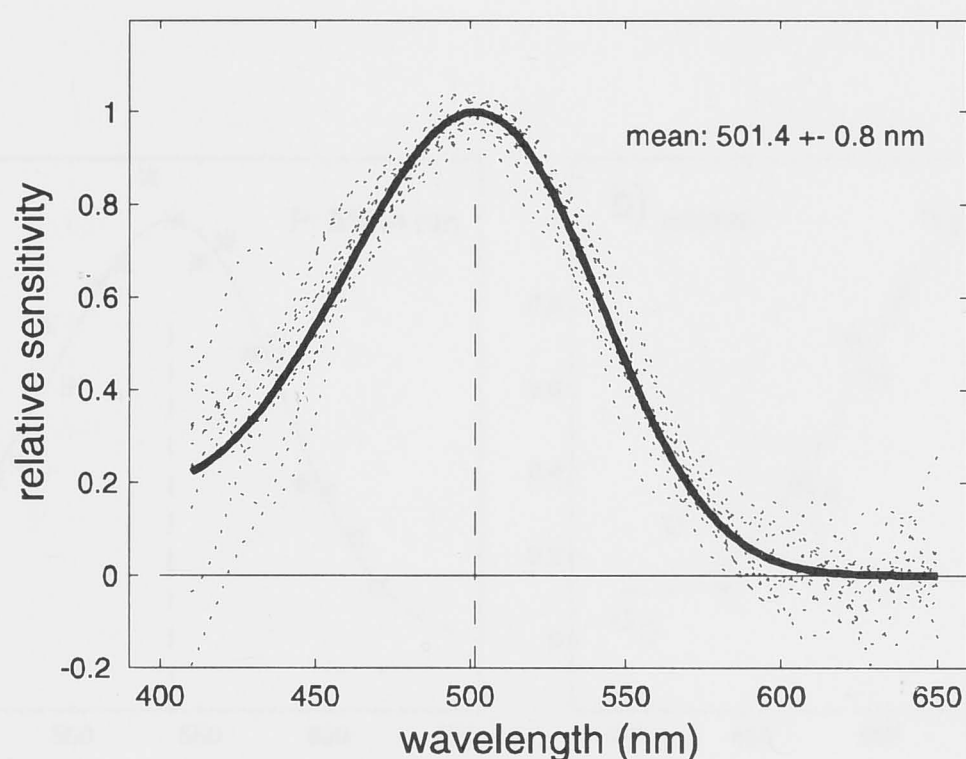


Figure 2-2: Microspectrophotometric measurements from 14 rod photoreceptors (dotted lines) and a retinal based (A1) nomogram (solid line) with a peak sensitivity at the mean for the 14 measurements.

Electroretinogram measurements

Middle wavelength cones

Figure 2-3 shows the spectral sensitivity curves obtained for 4 tammar wallabies when we recorded under conditions which were shown in other mammals to favour middle to long wavelength cones (e.g. Jacobs et al. 1985; Jacobs and Deegan II 1992). The circles and stars represent the computed sensitivity measurements. Only the stars were used in fitting a standard retinal based mammalian nomogram (Stavenga et al. 1993) to the data (solid curve). For all four animals the

peak sensitivity obtained fell within 1 nm of the mean at 539.4 nm. The largest standard deviation for the individual peak locations is 1.6 nm. The fits obtained for single nomograms are good and there is no indication that the data are the result of superimposed responses from two different pigment classes, or at least that one pigment is strongly dominating the response. When trying to fit two nomograms to the data, the fitting algorithm invariably fitted a positive and a negative nomogram, both located around the peak for the single nomogram, again suggesting that the recordings contained significant input from only one photoreceptor type.

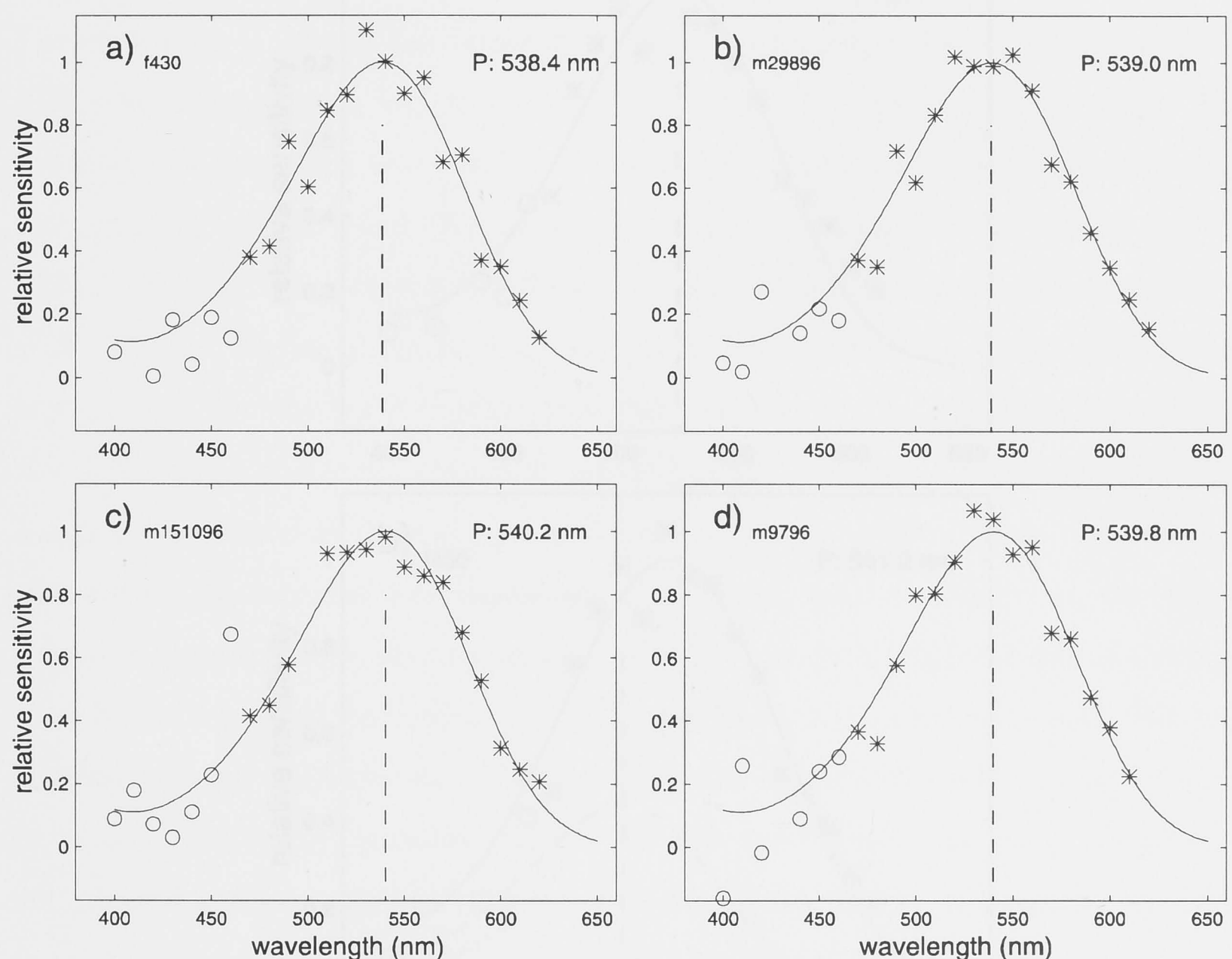


Figure 2-3: Spectral sensitivity measurements for 4 animals (female: a, males: b-d), under conditions that should favour the responses of M-cones, using an electroretinogram based procedure. Solid lines represent the best fitting retinal based nomogram. Only measurements above 460 nm (*) were used for the fits. The peak sensitivity of the fitted nomograms all fall within 2 nm at about 539 nm and the largest standard deviation for the peak is 1.6 nm.

Other photoreceptors

Animal f430 was also tested under conditions which should have favoured the S-cone response by reducing the stimulus contrast for the M-cones (539 nm) by a factor of nine to a final contrast of 9%. Any hypothetical short wavelength pigments would have been exposed to virtually normal contrast (see above). The results are shown in figure 2-4a, b.

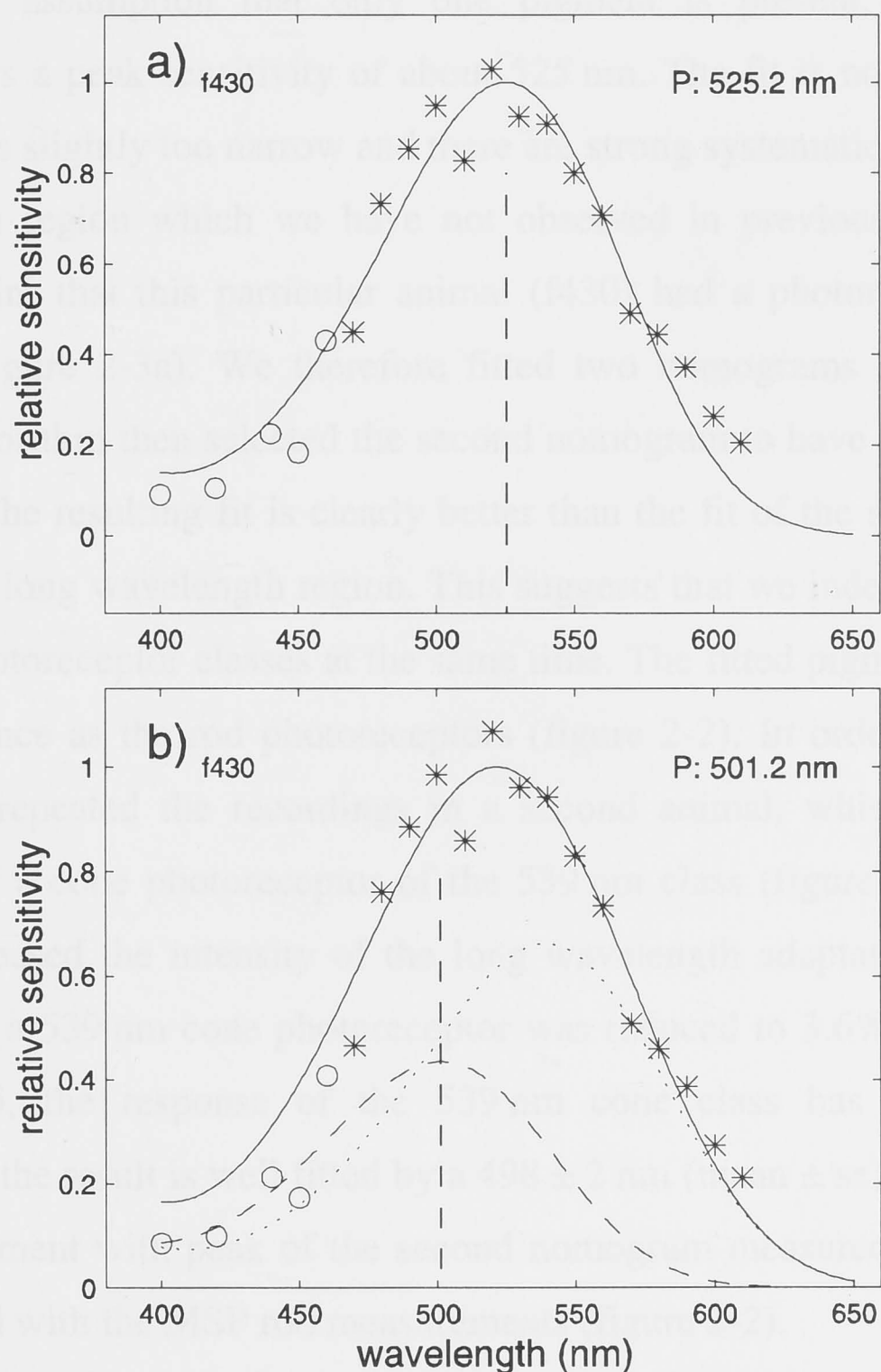


Figure 2-4: Spectral sensitivity measurements for one animal under conditions that should favour the S-cone response (see text for details). a) A single nomogram does not fit the data very well, especially in the long wavelength region. The nomogram also seems to be too

narrow. b) The same measurements fitted with a combination of two nomograms. The peak of one nomogram (dotted line) was fixed at 539 nm because the same animal was shown previously to have a photoreceptor of the 539 nm cone class (figure 2-3a). The algorithm selected a second nomogram (dashed line) with a peak sensitivity at about 501 ± 9 nm (mean \pm se). The combined sensitivity of the two nomograms (solid line) fits the data much better. The nomogram selected by the algorithm has the same spectral sensitivity as the rod photoreceptors (figure 2-2).

Under the assumption that only one pigment is present, the best fitting nomogram shows a peak sensitivity of about 525 nm. The fit is not ideal, however. The nomogram is slightly too narrow and there are strong systematic deviations in the long wavelength region which we have not observed in previous recordings. We knew at this point that this particular animal (f430) had a photoreceptor from the 539 nm class (figure 2-3a). We therefore fitted two nomograms and fixed one at 539 nm. The algorithm then selected the second nomogram to have a peak sensitivity at 501 ± 9 nm. The resulting fit is clearly better than the fit of the single nomogram, especially in the long wavelength region. This suggests that we indeed recorded from two different photoreceptor classes at the same time. The fitted pigment has the same spectral absorbance as the rod photoreceptors (figure 2-2). In order to confirm this conclusion, we repeated the recordings in a second animal, which had also been shown to possess a cone photoreceptor of the 539 nm class (figure 2-3d). This time though, we increased the intensity of the long wavelength adaptation such that the contrast seen by a 539 nm cone photoreceptor was reduced to 3.6%. As can be seen from figure 2-5, the response of the 539 nm cone class has now completely disappeared and the result is well fitted by a 498 ± 2 nm (mean \pm se) nomogram. This is in good agreement with peak of the second nomogram measured for animal f430 (figure 2-4b) and with the MSP rod measurements (figure 2-2).

When we used an even stronger long pass adaptation light, with 50% transmission at about 558 nm with an intensity of 1700 cd/m^2 , we were not able to produce any consistent response changes associated with the secondary light. Under these conditions the contrast for a photoreceptor at 500 nm, as measured above,

would have had its stimulus contrast reduced by a factor of 6 to about 7% (without secondary lights), whereas a blue pigment of about 440 nm peak sensitivity would still have seen about 70% contrast.

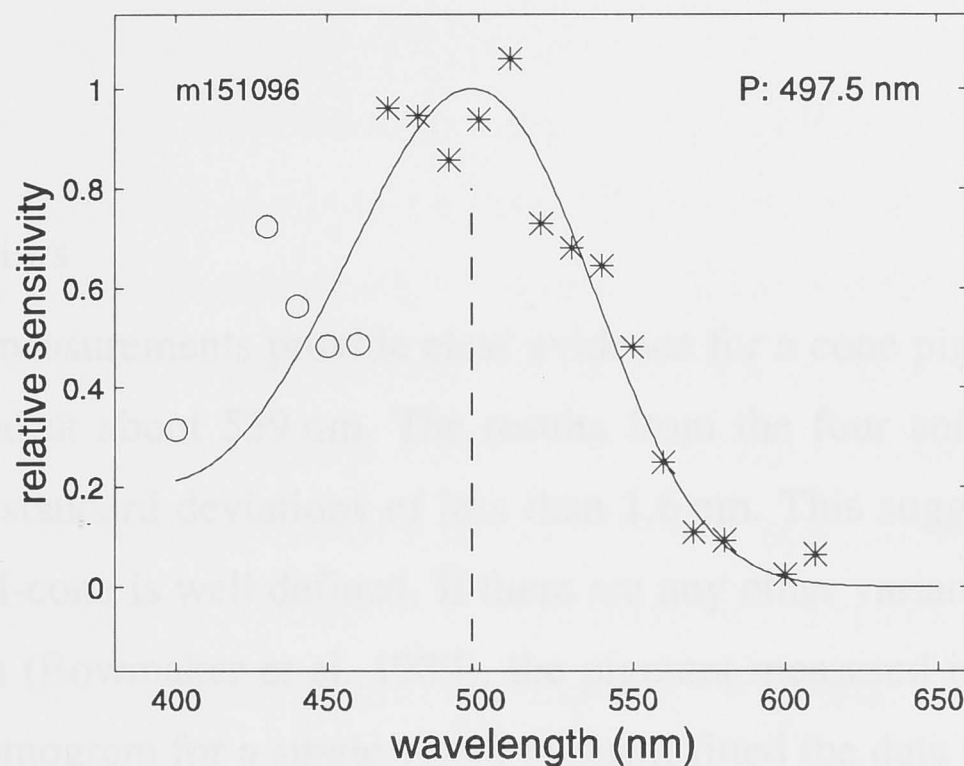


Figure 2-5: Spectral sensitivity measurements under strong concurrent long wavelength adaptation for an animal that has been shown to have a cone pigment from the 539 nm cone class (figure 2-3c). The responses of the 539 nm cone class have been completely suppressed by the adaptation light and the data are well fitted by a single nomogram with a peak sensitivity at about 498 ± 2 nm (mean \pm se). This is the same spectral sensitivity as has been found for the rod photoreceptors (figure 2-2) and for the second fitted nomogram in figure 2-4b.

Discussion

Rod photoreceptors

The MSP measurements show that the wallabies have a standard mammalian rhodopsin with an peak absorption at about 501 nm (Lythgoe 1972). This measurement could be confirmed by the ERG recordings where we have two

estimates of peak absorption at 498 and 501 nm respectively. The close correspondence between the MSP and the ERG recordings supports the impression of a human observer that the cornea and the lens in the tammar wallaby are colourless. The MSP measurements were not influenced by these structures, while the ERG recordings could potentially have been, yet the two methods both produced the same result.

Cone photoreceptors

The ERG measurements provide clear evidence for a cone pigment with a peak sensitivity located at about 539 nm. The results from the four animals are in close agreement, with standard deviations of less than 1.6 nm. This suggests that the peak location of the M-cone is well defined. If there are any other variants to this pigment in the population (Bowmaker et al. 1980), the pigment measured is clearly the most common. The nomogram for a single visual pigment fitted the data well in every case and there is no suggestion that the nomograms were too narrow as would be the case if we recorded from two cone populations at the same time. We were also not able to fit a combination of two nomograms to the recordings without one being attributed a negative absorbance by the fitting algorithm. This again suggests that there is only one middle to long wavelength pigment in this species. There is of course the possibility that we missed a potential second middle to long wavelength pigment with a very low density. However, the data in the long wavelength region are accurate, which would not have been the case if the measurements were significantly influenced by a second pigment.

Our attempts to find a short wave-length sensitive cone pigment were unsuccessful. We were able to suppress the responses of the M-cones to such a degree, that we either picked up a faint combined signal from the rods and the M-cones (figure 2-4a, b), or even completely abolished the M-cone response and only recorded a weak rod signal (figure 2-5). Nonetheless, in both these recordings there is no sign of any influence of a prospective S-cone. When we suppressed the rod signal even further by using a strong long pass adaptation light with 50% transmission at

560 nm, we failed to produce any consistent changes in the response level associated with the secondary lights at all.

There are several possible explanations for this result. a) Since we used a halogen light source, the monochromatic test lights in the blue region of the spectrum were weaker than in the green-red area and would have reduced our accuracy in the blue range of the spectrum. Above 450 nm, however, we arrived at good, repeatable estimates for the M-cones and thus must have had enough light to at least detect the presence of an S-cone class, unless its peak fell at wavelength shorter than 400 nm. b) A second explanation is concerned with the density of the S-cones, which may be so low that their responses simply disappear in the recording noise. We have recently concluded an immunocytochemical study of the tammar retina and found clear evidence for two distinct cone sub-populations (Hemmi and Grünert submitted). One of the two populations was labelled by the antibody JH455 which usually labels the short wavelength sensitive cones in other mammalian retinæ (Wang et al. 1992; Goodchild et al. 1996; Sandmann et al. 1996). The two sub-populations had very different spatial density distributions, but both cone classes were present throughout the retina. In the area of the retina involved in the ERG recordings, the S-cones candidates have a density of 500 to 1000 cell/mm², or roughly 5 to 10% of the M-cone density. We therefore think that after abolishing the responses from the M-cones we should have been able to record some responses from these putative S-cones. c) The immunocytochemical study suggests yet another possible explanation for the failure to record S-cone activity even after abolishing M-cone responses. We noticed that all suspected S-cones could also be labelled with the antibody against M-cones. This raises the possibility that the S-cones also contain a significant amount of M-cone pigment in this species. There have indeed been suggestions for other mammals that there are cones that express two different visual pigments in the adult animal, although only in limited areas of the retina (e.g. Röhlich et al. 1994). If this were in fact the case in the tammar wallaby, it would mean that by adapting the M-cone pigment we also adapted the S-cones and as a consequence strongly reduced their responses. Two visual pigments in one cone population would not preclude the animal from having dichromatic colour vision, however, but would reduce the signal

to noise ratio in the chromatic channel. d) Our ERG results can of course also be taken to mean that wallabies do not have S-cones. This conclusion, however, does not agree with the immunocytochemical results and more importantly is at odds with recent behavioural evidence, which clearly shows that the wallabies are dichromats with a null-point at about 485 nm (Hemmi submitted). The results from the behavioural study can only be explained if we postulate the presence of a short wavelength sensitive cone class with a peak sensitivity of about 420 nm. For the same reason, the behavioural results also rule out the possibility that the 'rod' responses measured in our ERG experiments were produced by an unusual S-cone population with a peak sensitivity around 500 nm.

Another issue is the presence of retinal oil droplets in the retina of the tammar wallaby, as has been found in other marsupials (Hoffmann 1876-77; O'Day 1935). Oil droplets are often discussed in relation to colour vision (e.g. Muntz 1972; Jacobs 1981). In the tammar, however, these oil droplets are all transparent to the human observer and in our immunocytochemical study we found that all cones contain oil droplets (Hemmi and Grünert submitted). Also, the good fit between the nomograms and the M-cone responses show that these responses are not strongly distorted by the oil droplets. For these reasons, we do not think that oil droplets are involved in colour vision in this species. Their role is more likely to increase the light sensitivity of the cone photoreceptors (Young and Martin 1984).

Comparison with the American opossums

The situation in the tammar wallaby is comparable to that reported for the opossums (Jacobs 1993), showing the same conflicting information between the ERG, the immunocytochemical and the behavioural results. As in the tammar, the putative immunolabelled S-cone population in the South American opossum has a very low density of about 300 cells/mm² (Ahnelt et al. 1995), but it is unknown whether the S-cones found in the opossum also double label with the antibody against the M-cones.

Conclusion

In this study we showed that the tammar wallaby has a standard mammalian rhodopsin at about 500 nm and we found one cone photoreceptor with a peak spectral sensitivity at about 539 nm. Our failure to find a short wavelength sensitive cone class is most likely attributable to the technique used to measure spectral sensitivity. There is a clear need to undertake a microspectrophotometric study or to make intracellular recordings in both the tammar wallaby and the American opossums to actually measure the spectral sensitivity of the putative S-cone photoreceptors with a method that is not limited by the relative density of the photoreceptors. Such a study could also clearly confirm whether these cones do in fact contain two visual pigments or not.

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From physiology to anatomy

In chapter 2 evidence was presented for the presence, in the tammar wallaby retina, of middle wavelength sensitive cones (M-cones) with a peak spectral sensitivity at 540 nm and a rod photoreceptor with a peak spectral sensitivity at 500 nm. Both these pigments are not unusual for a mammal (Lythgoe 1972; Jacobs 1993). Surprisingly, however, there was no evidence for the presence of a short wavelength sensitive cone (S-cone), even though similar studies showed the presence of small S-cone populations in a range of mammals (Jacobs 1993). It is intriguing that the same approach has also failed in the only other marsupial in which it was attempted, the Virginia opossum (*Didelphis virginiana*). In order to set this result in perspective the next step was an immunocytochemical study using two antisera that have been found to label M and S-cones in a range of mammals (Wang et al. 1992; Goodchild et al. 1996; Sandmann et al. 1996). It was reasoned, that, as long as the wallaby does not have a particularly unusual S-cone pigment, this method should be able to show its presence, although not its functionality, without the problems created by isolating potentially small signals from a large noisy background in electrophysiological recordings. This study also provided valuable information about the spatial distribution of the different photoreceptors across the retina. From this topographic information clear hypotheses can be formulated about functional aspects of different parts of the retina.

Abstract

Chapter 3

Distribution of photoreceptor sub-populations in the retina of a marsupial, the tammar wallaby (*Macropus eugenii*)

J.M. Hemmi, U. Grünert

Abstract

Mammalian retinæ generally contain low numbers of short wavelength sensitive cones (S-cones) and higher numbers of middle to long wavelength sensitive cones (M-cones). Some recent studies found topographic differences between the different photoreceptor sub-populations and in some instances between photoreceptors and ganglion cells. To investigate this question further, we constructed topographical maps of the different photoreceptors found in an Australian marsupial, the tammar wallaby. We used two polyclonal antibodies that have been shown to label S-cones (JH455) or M-cones (JH492) in a range of mammals. In the tammar wallaby, the antibodies clearly distinguish two cone populations. JH455 recognises a small subset of cones (S-cones) with a density of less than 500 cells/mm² in the ventral retina. Their density increases towards the dorsal retina to about 1,300-2,000 cells/mm². JH492 recognises all remaining cones (M-cones), but also faintly labels most cone cells recognised by JH455. The distribution of M-cones, unlike that of the S-cones shows a clear horizontal streak of high cell density through the central retina, just like the ganglion cells. Unlike the ganglion cells, however, the M-cones do not peak in the temporal retina but show a very broad peak (12,000-18,000 cell/mm²) in the central or even slightly nasal retina. Based on our findings, the retina of the tammar can be divided into three distinct regions: Firstly, the dorsal retina, which has a low ganglion and low cone cell density but a high percentage of S-cones (30%), is thought to provide good spectral sensitivity; Secondly, the central horizontal band of retina, which has a high ganglion and high cone cell density and therefore provides good spatial resolution; And thirdly, the ventral retina, which has a low ganglion cell but high cone cell density with few S-cones (5%) and is therefore thought to have a high spatial sensitivity but low acuity.

Introduction

The identity and distribution of different photoreceptor sub-populations have recently been studied in a range of mammals using immunocytochemical methods (Müller and Peichl 1989; Wikler and Rakic 1990; Petry et al. 1993; Juliusson et al. 1994; Röhlich et al. 1994; Szél et al. 1994; Ahnelt et al. 1995; Famiglietti and Sharpe 1995; Peichl 1997). The emerging basic organisation for most of these mammals is a low density of short wavelength sensitive cones (S-cones) combined with a much higher density of middle wavelength sensitive cones (M-cones; Szél et al. 1996; Peichl 1997). The topographies of the different photoreceptor populations are variable. In the tree shrew retina for instance, the two cone populations have a similar distribution, which in turn is clearly different from the rod and the ganglion cell distribution (Müller and Peichl 1989; Petry et al. 1993). In the South American opossum, the M-cones without oil droplets show the same general topography as the ganglion cells, whereas other cone classes, double cones and single cones with oil droplets have a clearly distinct topography (Ahnelt et al. 1995). The functional significance of these different distributions is still not clear, but might reflect selective pressures to increase sensitivity (Ahnelt et al. 1995).

In a number of other species, such as the mouse, the rabbit and the guinea pig, there is even a strong topographic separation between the M and S-cones (Röhlich et al. 1994). Szél et al. (1996) concluded that the ventral S-cone fields (which do not contain any M-cones in these mammals) are rudimentary areas conserving an ancestral stage of photopigment evolution.

Whereas the ganglion cell topography in a wide range of marsupials has been well documented (Hughes 1977; Hokoç and Oswaldo-Cruz 1979; Tancred 1981; Beazley and Dunlop 1983; Wong et al. 1986; Schmid et al. 1992; Dunlop et al. 1994), no such information is available for their photoreceptors, with the exception of the South American opossum (Ahnelt et al. 1995). We performed an immunocytochemical study in the tammar wallaby which is a kangaroo like marsupial that inhabits parts of southern Australia, in order to remedy this lack of information for at least one Australian marsupial.

While the tammar is distantly related to the American opossums, it represents an order, the Diprotodonta, which underwent a strong radiation, producing a wide range of specialised species. The wallabies therefore provide information from a unique, highly evolved mammalian order, which can be compared to the data from eutherian mammals as well as the American opossums.

The retina of the tammar wallaby contains oil droplets and double cones, features that have been shown a long time ago to be present in marsupials (e.g. Hoffmann 1876-77; O'Day 1935). Oil droplets and double cones are also found in birds and reptiles, but are absent in eutherian mammals. The ganglion cell topography of the tammar wallaby shows a clear horizontal streak of high cell density through the central retina. The density along this streak peaks in the temporal retina where it forms a clear area centralis (Tancred 1981; Wong et al. 1986). The ventral retina is strongly pigmented and forms a clear contrast to the lightly pigmented dorsal retina. The retina of the tammar, does not have a tapetum. Its retinal magnification factor is 0.16 mm/deg (Hemmi and Mark 1998).

To differentiate between the different cone populations, we used two antibodies which are specific for different cone subclasses. The results of our study provides important comparative data needed to improve our understanding of retinal topography. It may also help to further our understanding of the evolution of colour vision.

Materials and Methods

Animals

All retinae used in this study came from adult male and female tammar wallabies (*Macropus eugenii*) weighing between 2 and 6 kg. The animals came from a breeding colony, used for experimental purposes and were kept in small social groups in outdoor paddocks. The animals were euthanased by an overdose of pentobarbitone (Nembutal) for unrelated physiological or anatomical experiments that left the eyes intact.

Tissue preparation

Retinae from 4 animals were fixed by ocular injection and transcardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 for 30 min. They were then removed and immersed into 4% paraformaldehyde for an additional 3.5-6 hours before being stored in PB at 4°C. Retinae from an additional three animals were removed immediately after death and the cornea was cut away. They were then immersion fixed for 4 hours in 4% paraformaldehyde in PB before being stored in PB at 4°C, with the exception of one retina which was stored in the fixative at 4°C for several months.

For whole mounts, retinae were flattened by radial cuts and photographed together with a scale-bar, before further processing. At the end of processing, the retinae were mounted in Mowiol (Hoechst, Germany; Harlow and Lane 1988). This mounting procedure kept shrinkage to a negligible level. In seven retinae shrinkage values were calculated. They were less than 2% in every case. The density measurements presented here were therefore not corrected for shrinkage.

For vertical and horizontal semithin sections (0.5 or 1 μm), blocks of about 1 mm² size and known eccentricity were cut out using a scalpel and dehydrated with ethanol. They were then transferred to acetone before being embedded in Medcast resin (Ted Pella). Shrinkage was not estimated.

Immunocytochemistry

Antibodies

The two affinity purified rabbit antisera, JH455 and JH492 which were used in this study were kindly supplied by Dr. J. Nathans. Antiserum JH455 was raised against a small section (33 amino acids) of the human blue pigment and JH492 was raised against a section (57 amino acids) of the human red pigment. For more details about production and selectivity testing see Wang et al. (1992). These antibodies have been found to selectively label the short wavelength sensitive cones (S-cones, JH455) and the middle to long wavelength sensitive cones (M-cones, JH492) in several mammalian species (Wang et al. 1992; Goodchild et al. 1996; Sandmann et al. 1996;

Chan and Grünert 1998). Antiserum JH492 was diluted to 1:50,000 and the antiserum JH455 was used at a dilution of 1:100,000 for whole mounts and 1:20,000 for horizontal sections.

Immunostaining

Immunocytochemistry was performed on retinal whole mounts as well as radial and horizontal semithin sections (i.e. perpendicular and parallel to retinal layers respectively). Whole retinae were processed free-floating at room temperature. They were immersed into 30% sucrose overnight for cryoprotection and then shock frozen and thawed three times to improve antibody penetration. They were then preincubated in 10% bovine serum albumin (BSA) and 0.5% Triton X-100 in phosphate buffered saline (PBS). Afterwards, the tissue was placed for three days at room temperature into the primary antibody solution containing one or the other antibody at the above dilutions in 1% BSA, 0.5% Triton X-100, 0.05% sodium azide in PBS. A secondary antibody coupled to HRP (Jackson goat α -rabbit) and diluted 1:300 in 1% BSA and 0.5% Triton X-100 in PBS was used to detect the primary (2 hours). The binding sites were then visualised by 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 . In four retinae 0.05% $NiCl_2$ was also added.

Semithin (0.5 or 1 μm) horizontal or vertical sections were cut from the resin embedded blocks and mounted alternately onto gelatinised slides so that consecutive sections, could be stained with different antibodies. For both horizontal and vertical sections the embedding media was first removed by immersing the slides for 10-15 minutes into alcoholic solution of sodium hydroxide after which they were rinsed in ethanol followed by distilled water. The sections were then incubated for 30 min in 1% normal goat serum (NGS) and 1% BSA in Tris-phosphate buffered saline (TPBS). The primary antibody, diluted in a solution of 1% NGS and 1% BSA in TPBS was then applied overnight at room temperature. A secondary antibody (Jackson, α -rabbit) coupled to HRP and diluted 1:300 in 0.1% BSA in TPBS was applied for one hour to detect the primary. The binding product was then visualised by 0.05% DAB and 0.01% H_2O_2 . No staining was observed when the primary antibody was omitted.

Removal of the pigment epithelium

In the tammar wallaby the pigment epithelium (PE) sticks firmly to the photoreceptor outer segments, even in the unfixed retina, and cannot be removed without substantial damage to the photoreceptors. In order to be able to count the stained and unstained photoreceptors in the highly pigmented ventral retina we therefore opted to bleach the PE by immersing the whole retina for 1-4 hours into a solution of 10% H_2O_2 in PBS with 10 drops of a 27% ammonia solution per 100 ml (modified from Petry et al. 1993). The bleaching, done after the immunostaining, did not seem to affect the stain but sometimes reduced the transparency of the final preparation and the visibility of the oil droplets.

Selectivity of staining

The staining of consecutive semithin horizontal sections with different antibodies allowed the same individual cone cells to be stained by both antibodies. The selectivity of the two antibodies could then be checked with camera lucida drawings and photomicrographs at 1250x magnification of identical regions in series of horizontal sections.

Topography of staining

An image analysis system (Magellan, Halasz and Martin 1984) was used to record the position of stained or unstained photoreceptors in the whole mounted retinae, using the optic disc and the strong pigmentation of the ventral retina as landmarks (the tammar retina is avascular). In some preparations, unstained photoreceptors could reliably be distinguished from rods based on the size of their inner segment and/or presence of an oil droplet. For three whole mounts, samples were taken at regular intervals throughout the retina. The sampling interval was adjusted to the density gradient by increasing sampling in regions where the photoreceptor density changed rapidly. The final sampling interval ranged between 0.5 and 2 mm. For the other retinae, counts were made at 0.5 to 1 mm intervals along ventral-dorsal transects (parallel to the vertical meridian) through the central part of

the retina. In some of the transects, rods were marked as well as cones at the same locations. Unstained photoreceptors were marked in the centre of their oil droplet or inner segment. Stained photoreceptors were marked in the centre of their oil droplet if it was clearly visible, or at the inner end of their outer segment. If there was uncertainty as to whether the position of the mark was inside the counting square or not, the photoreceptor was counted when it was located at the top or right margin of the counting square, but not if it was at the lower or left margin.

The sizes of oil droplets were estimated from one retina by moving a vertical line to both the left and the right side of each oil droplet. The distance between the two lines gave an estimate of the diameter of the oil droplet. The sizes were estimated every two millimetres (every millimetre in the centre) along a ventral-dorsal transect through the centre of the retina. At each retinal location a minimum of 10 cones stained with JH455 were marked and for each of these cones the nearest (judged by the centre of the oil droplet) oil droplet of an unstained cone was also measured.

Results

Differential staining and antibody specificity

The antibodies used in this study clearly enabled us to identify two different cone populations in the retina of the tammar wallaby. Figure 3-1a shows a 1 μm semithin section through the nasal tammar retina, treated with the antibody JH455 that has been raised against human S-cones. The outer segments of two cones are clearly labelled (arrows), while other cones, recognisable by their oil droplets, do not show any label (arrowheads). Figure 3-1b shows a nearby section (within 20 μm), that has been treated with JH492 which was raised against human L-cones. All cone outer segments in this section are immunopositive. After analysing a number of whole mounts treated with these antibodies, it was clear that JH455 is very selective and only stains a small sub-population of all cones. In contrast, we were not able to find any unlabelled cones in whole mounts treated with JH492, which seemed to label all cones. Unlabelled cones are usually very easy to see because of their oil droplets. To make sure we did not miss any unlabelled cones that were difficult to see or that

did not contain an oil droplet, we cut series of horizontal sections at 0.5 or 1 μm thickness through the outer segments. Alternate sections were then treated with either JH455 or JH492. This allowed us to label the same individual cone outer segments with the different antibodies and therefore check their specificity.

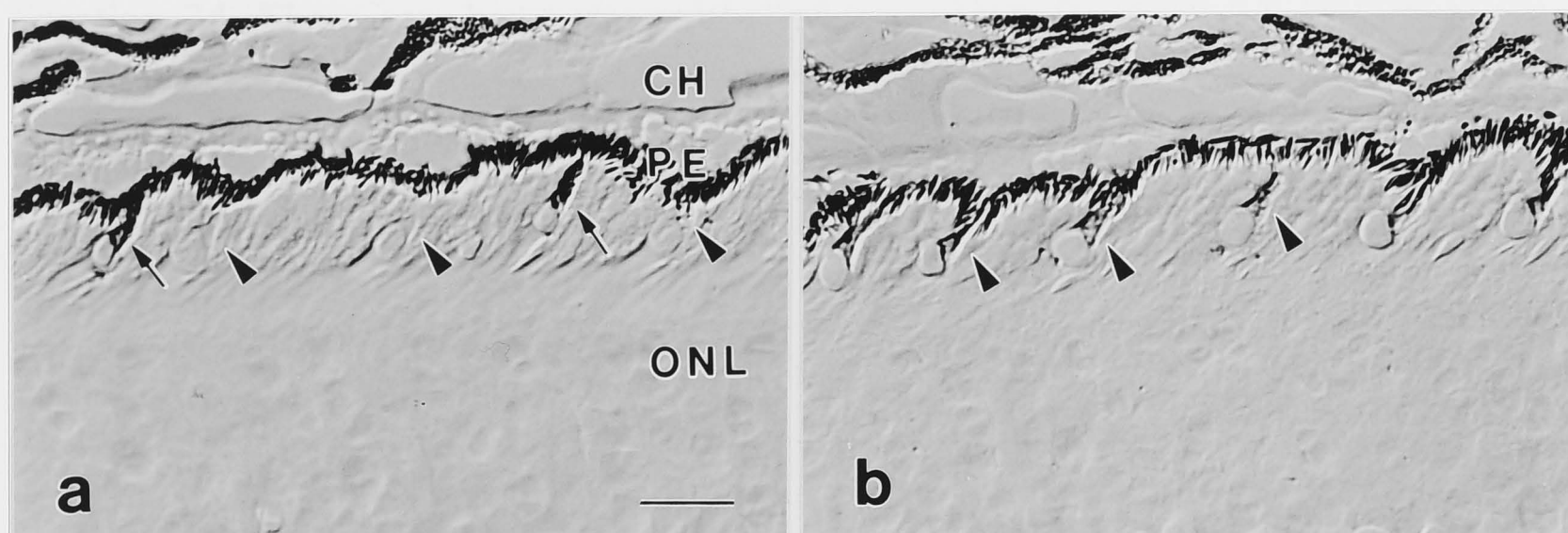


Figure 3-1: Light micrographs showing vertical 1 μm semithin sections through nasal retina of the tammar wallaby stained with cone pigment specific antibodies. a) This section was treated with the antibody JH455 which was raised against human S-cones which stains only a fraction of the total cone population. The outer segments of two cones are clearly labelled (arrows), while other cones, recognisable by their oil droplets are unlabelled (arrowheads). b) A nearby section (within 20 μm), was treated with the antibody JH492 which was raised against human L-cones. The outer segments of all cones are immunopositive (arrowheads). Abbreviations: Choroid (CH), pigment epithelium (PE), outer nuclear layer (ONL). Scalebar: 10 μm .

Figure 3-2 shows examples of adjacent horizontal sections from the dorsal and ventral retina. The section shown in figure 3-2a is from the dorsal retina and was treated with JH455 which again, clearly recognised only a sub-population of all cones (arrows). Figure 3-2b shows an adjacent section, treated with JH492. JH492 recognised all cones, and there are no unlabelled outer segments visible. The three cones, which are marked by arrows in figure 3-2a are also marked by arrows in figure 3-2b. They are clearly labelled by JH492, even though the label is lighter than in

cones that were not marked by JH455 (arrowheads). Figure 3-2c and 2d show two adjacent sections from the ventral retina. The section in figure 3-2c was stained with JH455 and the section in figure 3-2d with JH492. Only two S-cones are labelled in figure 3-2c (JH455, arrows), both of which are also labelled in figure 3-2d with JH492 (arrows). Whereas a large number of unlabelled cone outer segments are visible in section 2c (arrowheads), no unlabelled cone outer segments can be found in section 2d. There are, however, unlabelled cone inner segments visible, the outer segments of which were found in adjacent sections.

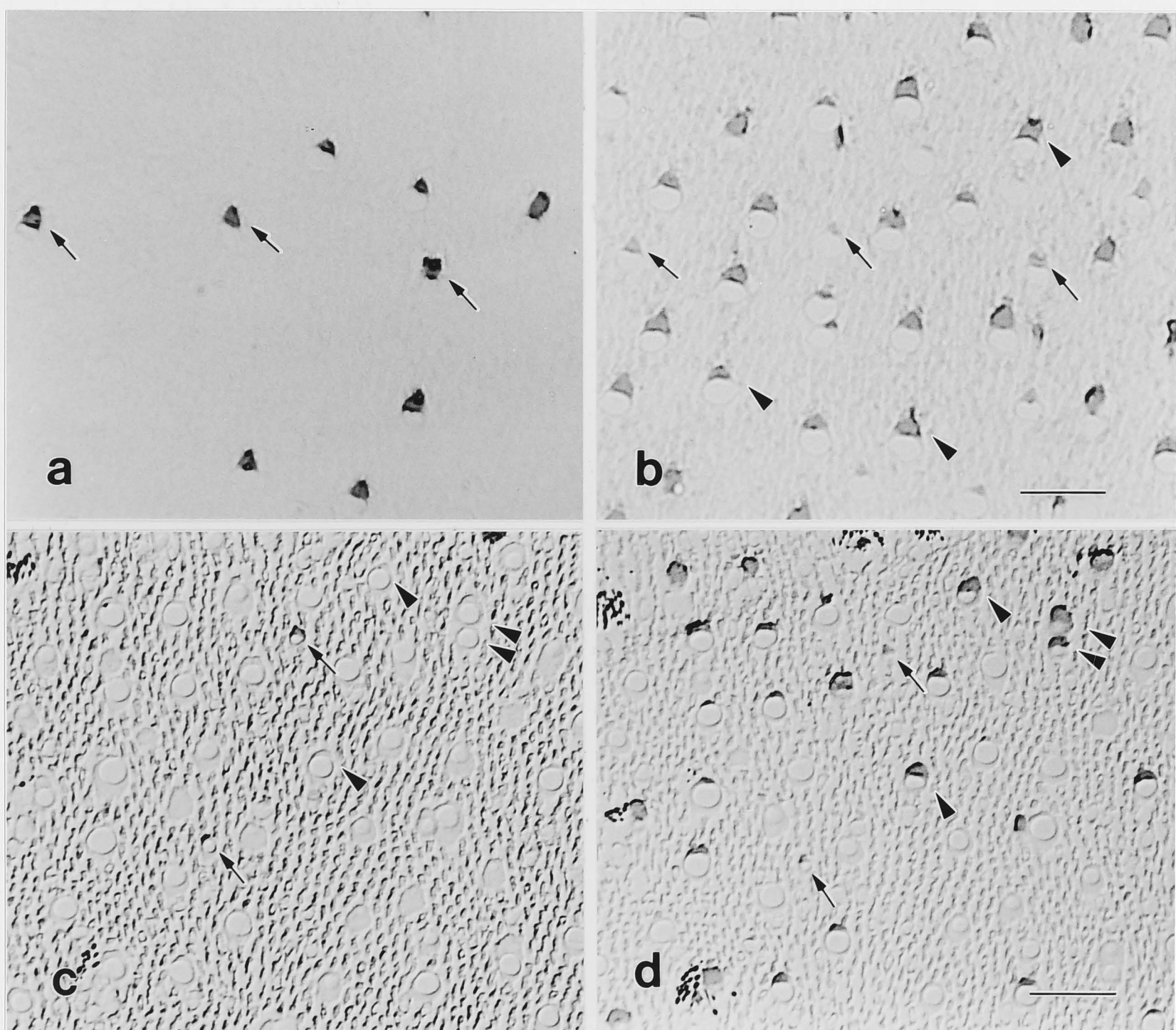


Figure 3-2: Horizontal 1 μ m sections through the level of the cone outer segments from dorsal (a, b) and ventral (c, d) retina. a) A section treated with JH455 shows a sub population of labelled cone outer segments (arrows). b) An adjacent section labelled with JH492. All cone outer segments are stained. The cones which are also stained by JH455 in figure 3-2a

are indicated by arrows. The staining of these double labelled cones (arrows) is usually weaker than that of cones only labelled by JH492 (arrowheads). c) As for section 2a, but a number of unlabelled cone outer segments are also marked (arrowheads). Note the smaller size of the labelled outer segments as compared to S-cones in figure 3-2a. d) An adjacent section to figure 3-2c, otherwise as for section 2b. Scalebar: 10 μ m.

In all retinal regions (i.e. dorsal, ventral and central), S-cones were found to be strongly labelled by JH455 and weakly labelled by JH492 (table 1a). These cones are therefore referred to as 'double labelled'. In the dorsal parts of the retinae from two animals (table 1a) all 87 S-cones found were double labelled by JH492. In the central retina, only 17 of 22 S-cones were double labelled and in three ventral region a total of 25 out of 34 S-cones were double labelled. In no instance could we clearly see an outer segment that was not labelled by the antibody JH492, although there were several instances where the label was very weak, approaching the background intensity. Double labelled cones in the central and ventral retina were more weakly labelled by JH492 than double labelled cones in the dorsal retina, whereas single labelled cones in all retinal regions were strongly labelled. The size of the outer segments in the ventral retina was clearly smaller in cones that were labelled by JH455 than in cones that were only labelled by JH492.

It is not clear whether the minority of cones that were labelled by JH455 (10%), but could not be confirmed to be labelled with JH492, were genuinely not double labelled, or whether it was just not possible to identify the label. The cone outer segments were often so closely embedded into the pigment epithelium (PE), that we only had very few sections to check for labelling, especially in the central and ventral retina. The oil droplets of cones that were labelled with JH455 often seemed to sit just adjacent to the PE with most of the outer segment surrounded by the PE. This meant that we could clearly make out the generally strong label of JH455, but the weaker label of JH492 could have been concealed by the PE.

Table 1: Summary of results from horizontal sections from 7 sample locations. The sampling location is given in mm relative to the optic disc along a vertical transect through the central retina. The table contains results from two different sampling strategies: a) Every S-cone that was found in a particular sample location was investigated for double labelling (see text for more detail). b) In 6 of these sections, we also traced all cone cells found in a randomly placed sampling grid from their inner to their outer segments. The number of cones found in each grid is given, as is the number of S-cones and the number of double cones. The percentage of double cones is given in relations to M-cones only. All cones found were labelled by at least one of the two antibodies (JH455; JH492) and all cones contained an oil droplet. All double cones were made up of two cones which were only stained by JH492.

Sample location	a)			b)			
	Sampled S-cones			Cone cells found in a randomly placed sample grid			
	total number	double labelled		grid size	cones found	S-cones	double cones
mm	nr.	nr. (%)		(μm^2)	nr.	nr. (%)	(%)
V ¹ -6.4	8	5 (63)		2500	31	2 (7)	3 (11)
V ² -5.1	18	13 (72)		2500	36	2 (6)	3 (10)
V ¹ -0.8	8	7 (88)		10000	134	5 (4)	11 (10)
C ¹ 1.8	14	13 (93)		7200	114	8 (7)	7 (7)
C ¹ 2.3	8	4 (50)					
D ¹ 13.3	38	38 (100)		9240	75	14 (19)	6 (11)
D ² 15.3	49	49 (100)		5000	55	17 (31)	3 (8)
	143	129			445	48 (11)	32 (9)

¹animal f912; ²animal m23497; Abbreviations: Ventral (V), central (C), dorsal (D)

In a number of retinal locations we marked out a grid (2500–10000 μm^2) and traced every cone within that grid from its inner to its outer segment through the different sections. This was done to confirm that all cone photoreceptors had an oil droplet and that all cones were marked by at least one of the two antibodies. From a total of 445 cones in two animals and six different retinal locations (table 1b) all

cones were marked by either one or both of the antibodies and every cone contained an oil droplet. In this sample of 445 cones there appeared to be 32 double cones, as judged by the fact that the inner segments of 2 adjacent cones were pressed flat against each other or that their inner segments were indistinguishable in these sections as was often the case. Double cones made up an average of 9% of all M-cones, a percentage that does not seem to change across the retina (table 1b). All 64 members of these double cones were stained exclusively by JH492 but not JH455.

In the following we will refer to cones labelled by JH455 as S-cones, regardless of whether the same cones were also recognised by JH492 or not. Cones that were labelled by JH492 but not by JH455 will be referred to as M-cones.

Topography of photoreceptors.

The retinal topography of the two cone sub-populations has been estimated from six whole mounts. S-cone numbers were always estimated from retinae stained with JH455. As previously mentioned, no unlabelled cones were observed in whole mounts treated with JH492. Because of this and the results from the horizontal sections, we assumed that counts made from retinae labelled by JH492 represent the total number of cones, including both M and S-cones. In four retinae treated with JH455 we estimated the M-cone density by counting unlabelled cones. Unlabelled cones could be recognised by the presence of an oil droplet (all cones contain an oil droplet) and the size of their inner segments (figure 3-3). The photomicrograph shown in figure 3-3 was taken from a whole mount stained with JH455. The location is just dorsal of the visual streak (0.5 mm) in the central retina. The plane of focus is on the level of the inner segments. The black marks above some of the large inner segments (large arrows) are stained outer segments of S-cones. There are also a number of unstained M-cone inner segments visible (arrowheads). Rods can easily be recognised by their small inner segments (small arrows).

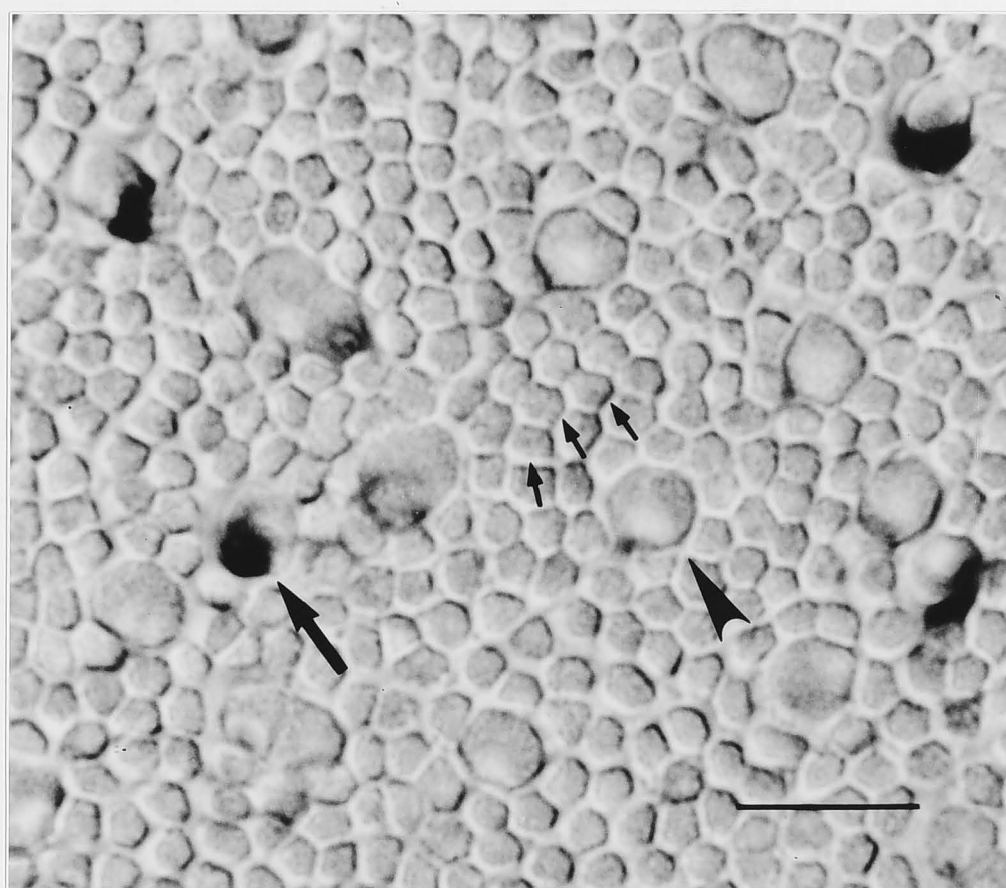


Figure 3-3: Light micrograph of a whole mount of a tammar wallaby retina stained with JH455, focussed on the level of the inner segments. The inner segments of cones labelled by JH455 (large arrows) and unlabelled cones (arrowheads), are easily distinguished from the smaller rod inner segments (small arrows). From such preparations the density of the rods and the two different cone types could be measured. Scalebar: 10 μ m.

Figure 3-4 shows examples of whole mounts stained with either JH492 (figure 3-4a, b, c) or with JH455 (figure 3-4d, e, f). The photomicrographs are taken from the following locations: dorsal (a, c), central (b, d) and ventral (e, f). Note the change in density for both cones across the different retinal locations. In figures 4d-f it is possible to see the oil droplets of unstained M-cones.

Figure 3-5 shows contour plots of the distribution of M-cones (a) and S-cones (b) of the animal m154r to give an overall impression of the cone distributions. The retina was stained with JH455 and M-cones were estimated from the unlabelled cones. Contour lines are plotted at 10% intervals between the lowest and highest densities measured. A thin-plate spline (Wahba 1990) was used to interpolate

between the measured locations and slightly smooth the resulting topography in order to make the graph easier to read. The topography of M-cones shows a clear horizontal streak of high cell density. Along this streak, the cell density changes only slowly, peaking in the central retina with about 12,500 cells/mm². From the streak, the density drops sharply towards the dorsal retina where it reaches its lowest levels of about 3,000 cells/mm². On the ventral side of the streak, the density increases again after an initial drop to reach quite a high value of 9,000 cells/mm². The S-cone distribution is markedly different (figure 3-5b). The overall density is much lower and steadily increases from about 300 cells/mm² in the ventral retina to about 1,500-1,600 cells/mm² in the dorsal retina. The S-cone density does not change significantly along the horizontal meridian.

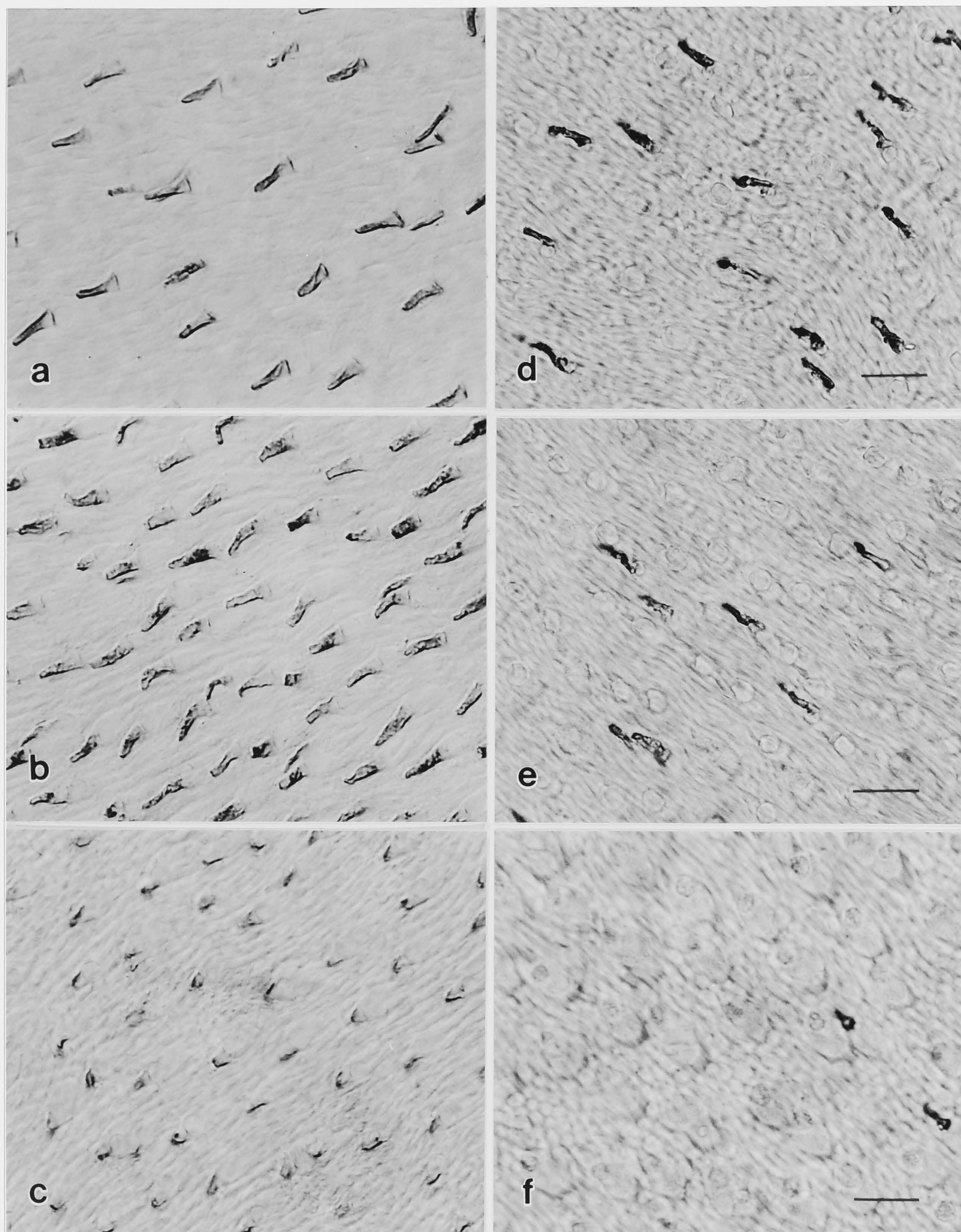


Figure 3-4: Photomicrographs of two whole mounts from tammar wallaby retinae stained with either JH492 (a, b, c) or JH455 (d, e, f). Samples 4a, d are taken from the dorsal retina, samples 4b, e from the central retina on the visual streak and samples 4c f are from the ventral retina. Note the oil droplets of unstained cones in the retina stained with JH455 (d, e, f). The cones stained with JH492 (a, b, c) have clearly a different distribution to the cones stained with JH455 (d, e, f). Scalebar: 10 μ m.

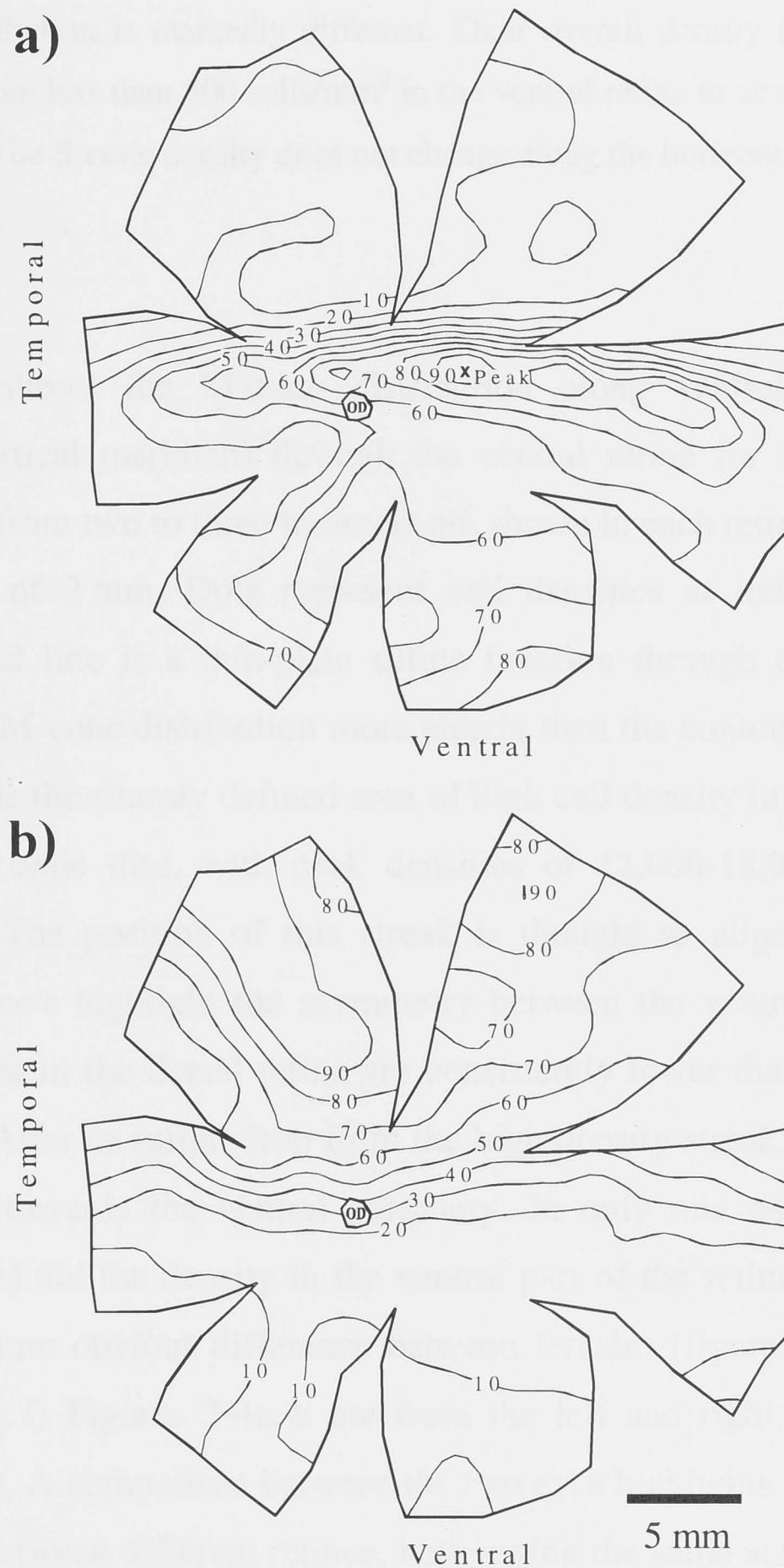


Figure 3-5: Contour density plots of the cone photoreceptors in a tammar wallaby. Contour lines are plotted at 10% intervals between the lowest and highest measured densities. a) Topography of M-cones. There is a clear horizontal streak of high density in the central retina from which the density drops towards both the ventral and dorsal retina, reaching its lowest level (3,000 cells/mm²) in the dorsal retina. The peak density (12,500 cells/mm²; marked by an x) is found in the centre of the streak. There is a strong asymmetry between the dorsal and ventral retina. The density in the ventral retina is much higher, and after a initial drop from the visual streak, it actually increases again towards the periphery to reach 9,000 cells/mm².

b) The S-cone distribution is markedly different. Their overall density is much lower and steadily increases from less than 500 cells/mm² in the ventral retina to about 1,300 cells/mm² in the dorsal retina. The S-cone density does not change along the horizontal meridian.

M-cones

Figure 3-6 shows the M-cone distribution along ventral-dorsal transects (parallel to the vertical meridian) through the central retina for five animals (six retinae). Densities from two to three transects are shown in each retina with a distance between transects of 2 mm. Dots represent cell densities at individual sampling locations. The solid line is a thin-plate spline function through these points. The transects show the M-cone distribution more clearly than the contour plots. The most distinctive feature is the sharply defined area of high cell density in the central retina just dorsal of the optic disc, with peak densities of 12,000-18,000 cells/mm² for different animals. The position of this streak is thought to align with the visual horizon. The transects highlight the asymmetry between the ventral and the dorsal retina. The densities in the dorsal retina are consistently lower than the densities in the ventral retina. After an initial drop from the high density streak, the cone density actually increases towards the ventral periphery. In only one animal, a very old female (figure 3-6e) did the density in the ventral part of the retina fall towards the periphery. There is no obvious difference between females (figure 3-6a, b, c, e) and males (figure 3-6d, f). Figures 3-4a, b are from the left and right eye of the same female respectively. A comparison between the two eyes highlights the fact that there is some variation between different retinae, both within the same animal and between animals. On the other hand, results along different transects in the same retinae are quite consistent.

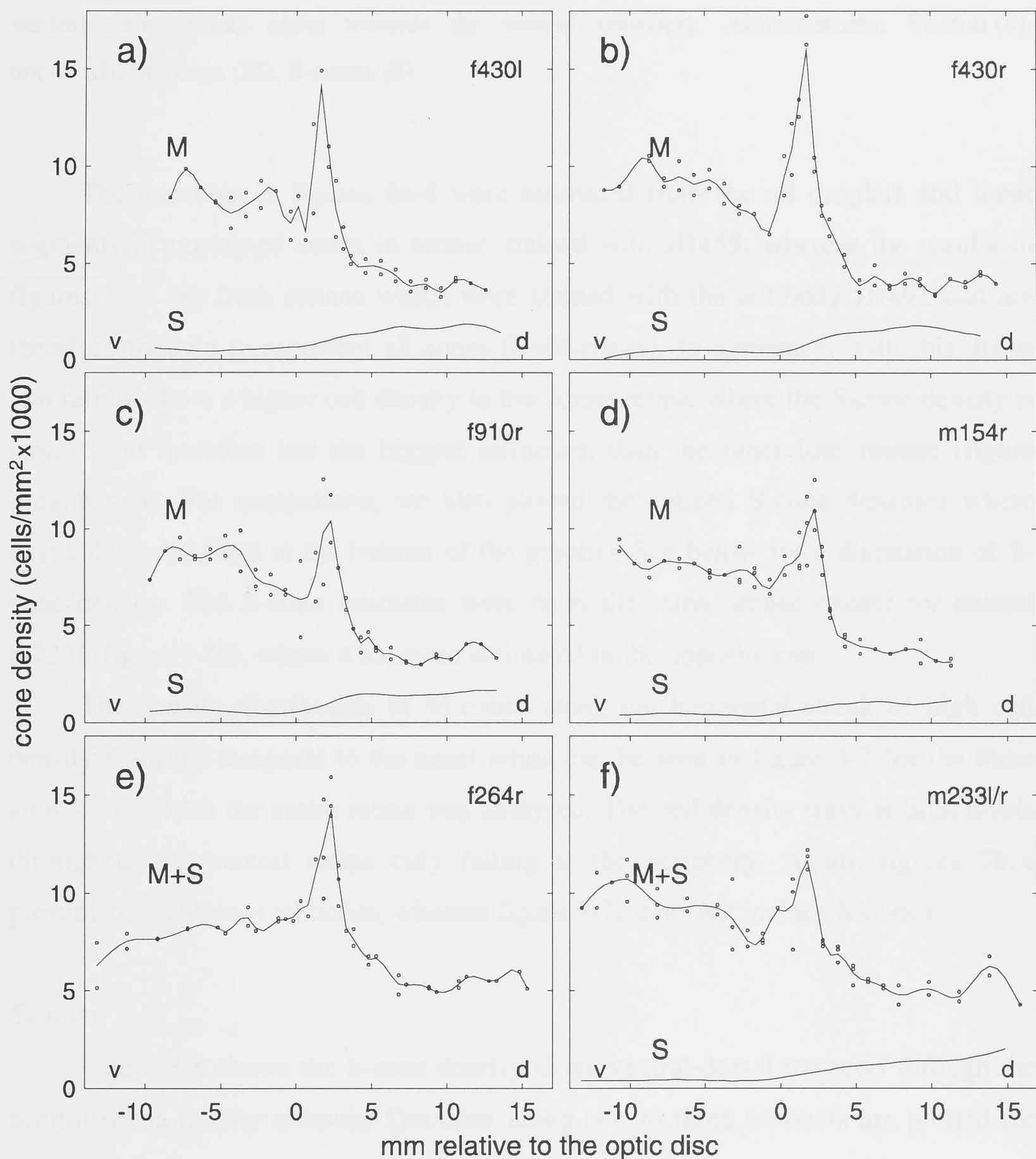


Figure 3-6: Density of M and S-cones along ventral-dorsal transects in five animals (six retinæ). Dots represent cell densities at individual sample locations and the solid line is a thin plate spline function through these data points. 6a, b are from the left and right eye of one female respectively. The densities along the transects in a-d were estimated by counting unstained photoreceptors in retinæ stained with the antibody JH455 (S-cones). The densities in 6e, f are from retinæ stained with the antibody JH492 (M-cones). The transects in 6a, b, c and e are from females and the transects in 6d, f from males. The density is lowest in the dorsal retina, peaks strongly in the central retina and after an initial decline towards the

ventral retina raises again towards the ventral periphery. Abbreviations: Ventral (v), dorsal (d), M-cones (M), S-cones (S)

The densities in figures 6a-d were estimated from the oil droplets and inner segments of unstained cones in retinae stained with JH455, whereas the results in figures 6e, f are from retinae which were stained with the antibody JH492 and are therefore thought to represent all cones (S+M-cones). In agreement with this, these two retinae show a higher cell density in the dorsal retina, where the S-cone density is largest and therefore has the biggest influence, than the other four retinae (figure 3-6a, b, c, d). For comparison, we also plotted the splined S-cone densities where available (solid lines at the bottom of the graphs). See below for a discussion of S-cone density. The S-cone estimates were from the same retinae except for animal m2331 (figure 3-6f), where it has been estimated in the opposite eye.

The density distribution of M-cones along the horizontal streak of high cell density from the temporal to the nasal retina can be seen in figure 3-7 for the three animals in which the entire retina was analysed. The cell density stays at high levels throughout the central retina only falling at the periphery. Again, figures 7b, c presumably represent all cones, whereas figure 3-7a does not include S-cones.

S-cones

Figure 3-8 shows the S-cone density along ventral-dorsal transects through the central retina in four animals. Densities along two to three transects are plotted for each retina. The distance between transects is 2 mm. The overall densities are much lower than for M-cones, with peak densities reaching about 1,300-2,000 cells/mm². Also, in sharp contrast to the M-cones, the S-cones have a low density throughout the ventral retina. The density starts to increase just dorsal of the optic disc to plateau at its highest level in the dorsal part of the retina. The density steadily increases through the central retina and there is no indication of a peak in this region.

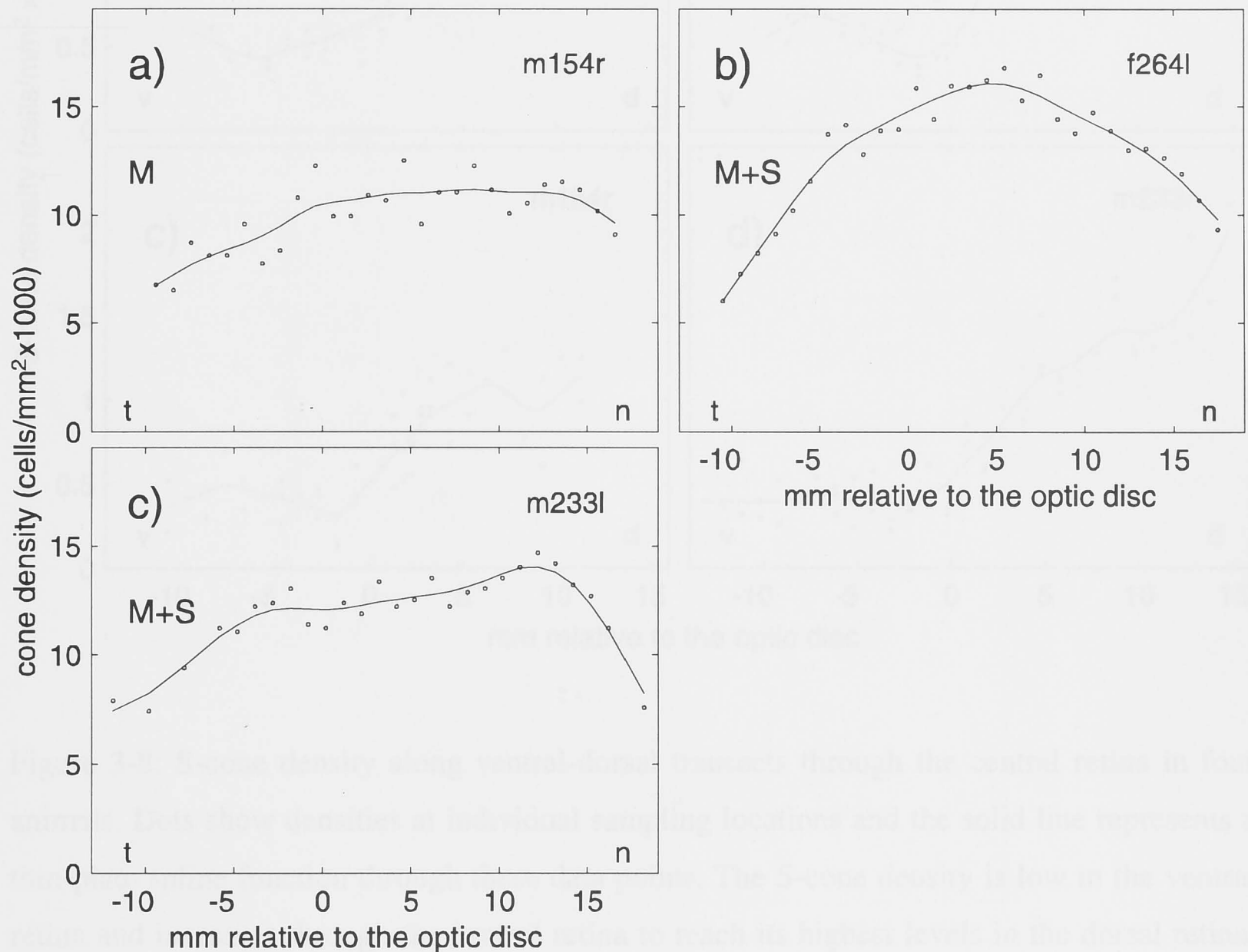


Figure 3-7: Density of M-cones along the horizontal streak of highest M-cone density for three tammar wallabies one female (7b) and two males (7a, c). Dots represent densities at individual sampling locations and the solid line is a thin-plate spline function through these data points. Transects 7b, c are estimated from retinæ stained with JH492 and therefore include S-cones. The transect in section 7a does not include S-cones, as only unlabelled cones were counted in a retina stained with JH455. The M-cone density changes only slowly along the horizon, showing a very broad peak in the central retina. Abbreviations: Temporal (t), nasal (n), M-cones (M), S-cones (S).

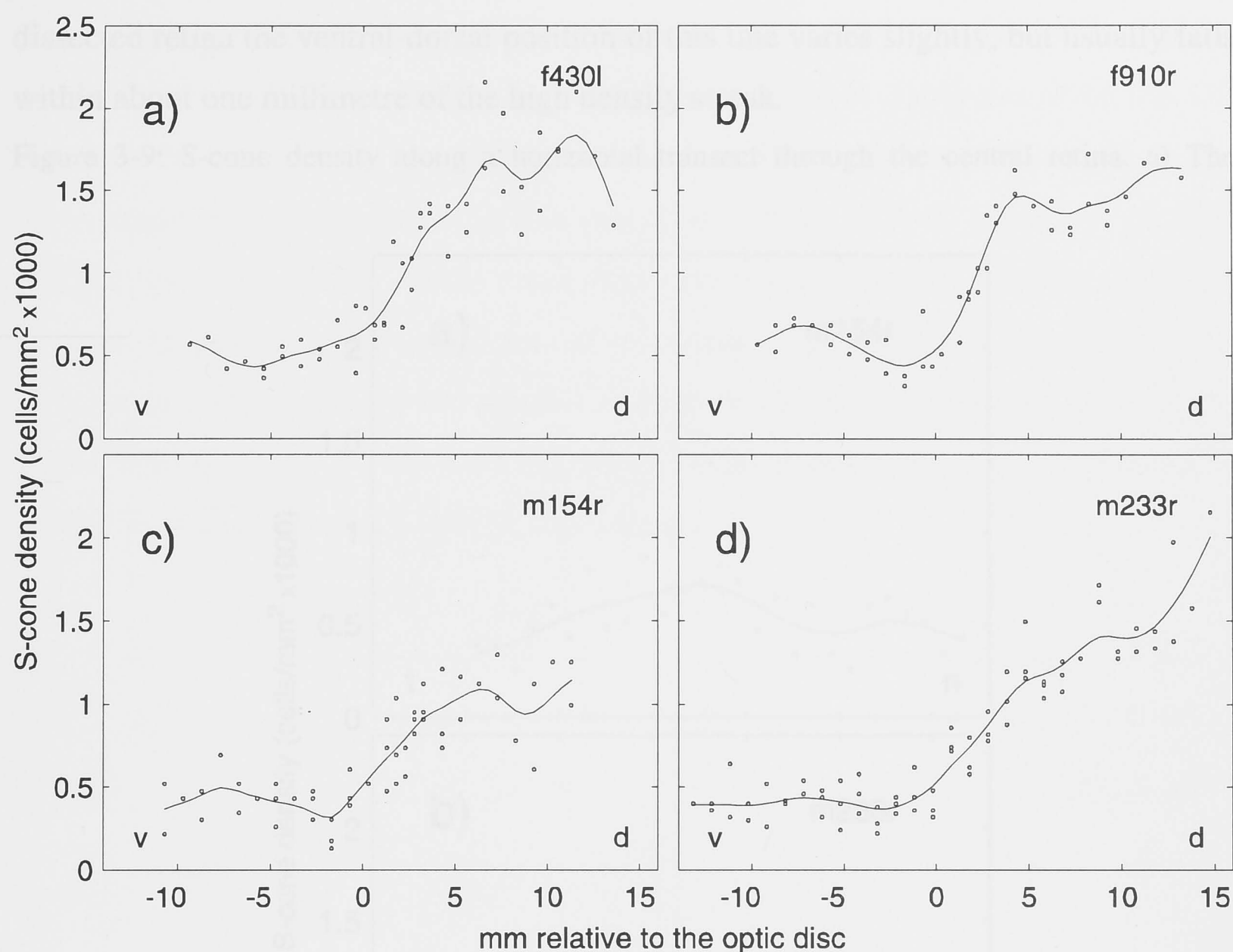
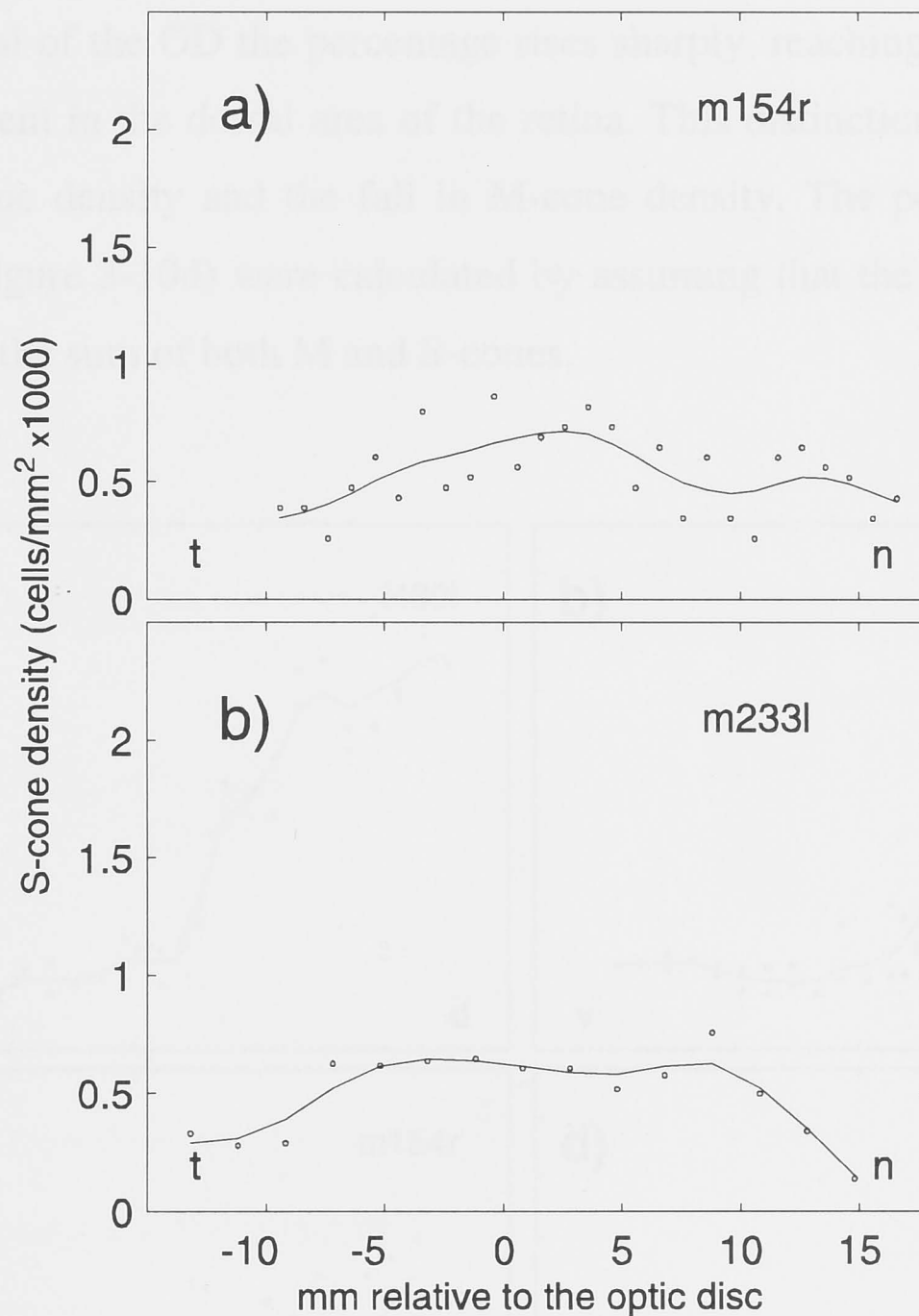


Figure 3-8: S-cone density along ventral-dorsal transects through the central retina in four animals. Dots show densities at individual sampling locations and the solid line represents a thin-plate spline function through these data points. The S-cone density is low in the ventral retina and increases through the central retina to reach its highest levels in the dorsal retina. Abbreviations: Ventral (v), dorsal (d).

The S-cone density along the horizontal meridian is very uniform, only slightly dropping towards both the temporal and nasal periphery (figure 3-9a, b). The densities in figure 3-9a are from the animal m154r where S and M-cones were measured in the same retina. This allowed us to measure the S-cone density exactly along the streak of highest M-cone density, which is thought to align with the visual horizon. Figure 3-9b is from animal m233l where M and S-cones were measured in different retinæ. The densities were therefore measured along the ventral-dorsal change in pigmentation which lies parallel to the highest M-cone density. In the

dissected retina the ventral-dorsal position of this line varies slightly, but usually falls within about one millimetre of the high density streak.

Figure 3-9: S-cone density along a horizontal transect through the central retina. a) The



transect is located on the streak of highest M-cone density counted in the same retina. b) Transect along the streak of highest M-cone density. Dots show densities at individual sampling locations and the solid line represents a thin-plate spline function through these data points. There is little change in the S-cone density along the horizon. Abbreviations: Temporal (t), nasal (n).

Percentage of S-cones

The percentage of S-cones of the entire cone population exemplifies the different distributions between the S-and M-cones (figure 3-10a-d). Throughout the

ventral retina the S-cone percentage is very low, about 5.4% (mean of 4 animals, ventral of the OD). The percentage stays low until about 2 mm dorsal of the OD where the M-cone density was shown to be highest, indicating that the S-cones mirror the increase in M-cone density in this part of the retina. Beyond about two to three millimetres dorsal of the OD the percentage rises sharply, reaching its highest levels of about 30 percent in the dorsal area of the retina. This distinction reflects both the increase in S-cone density and the fall in M-cone density. The percentages for the animal m233l (figure 3-10d) were calculated by assuming that the cones labelled by JH492 represent the sum of both M and S-cones.

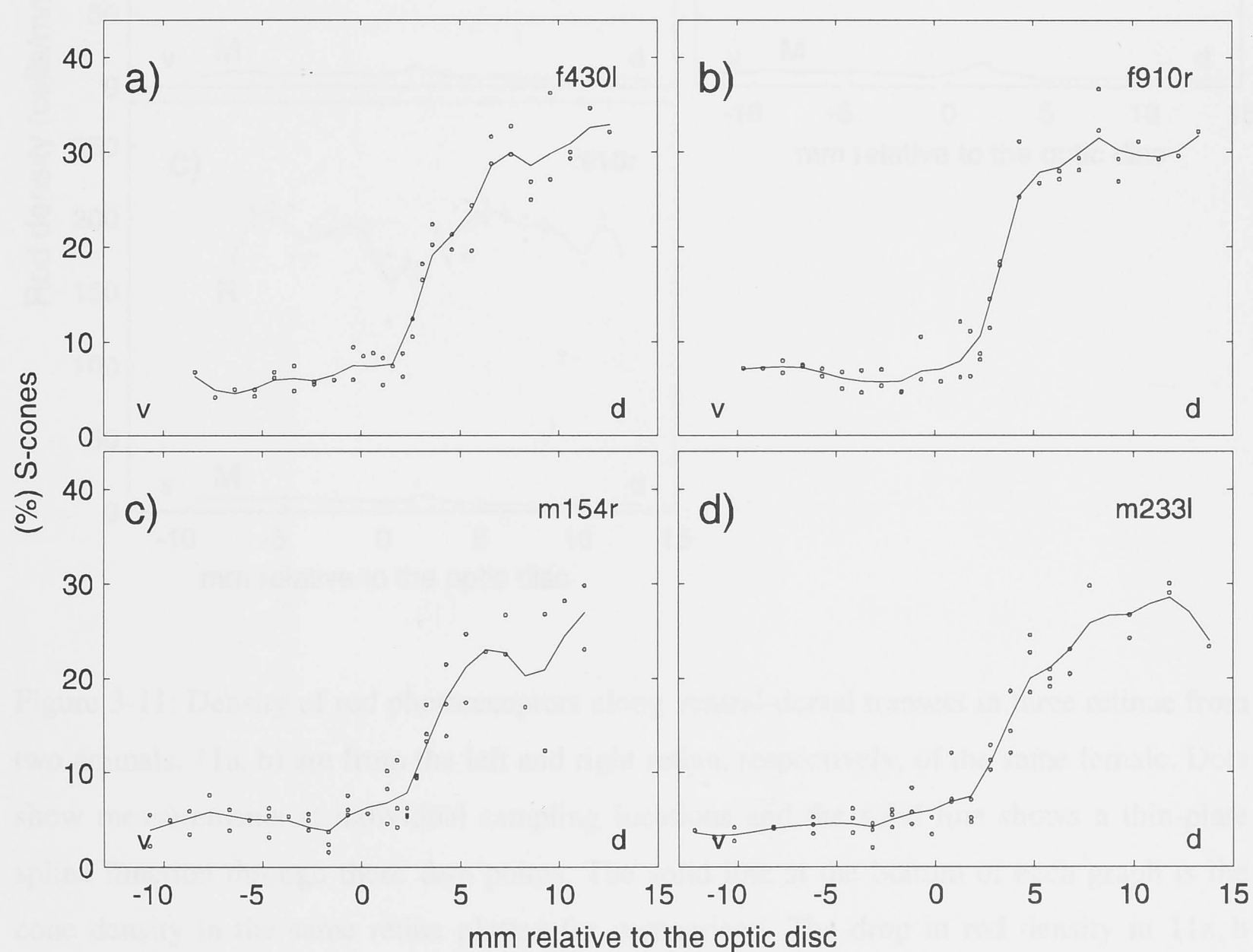


Figure 3-10: S-cones as a percentage of the total cone population along ventral-dorsal transects in four animals. Dots show measurements at individual sampling locations and the solid line shows a thin-plate spline function through these data points. a-c) The total number of cones was calculated by summing S and M-cones. d) S and M-cones were estimated in opposite retinæ. The percentage of S-cones is low throughout the ventral retina and only

starts to increase towards the dorsal retina after the M-cones have reached their highest density in the central retina. Abbreviations: Ventral (v), dorsal (d).

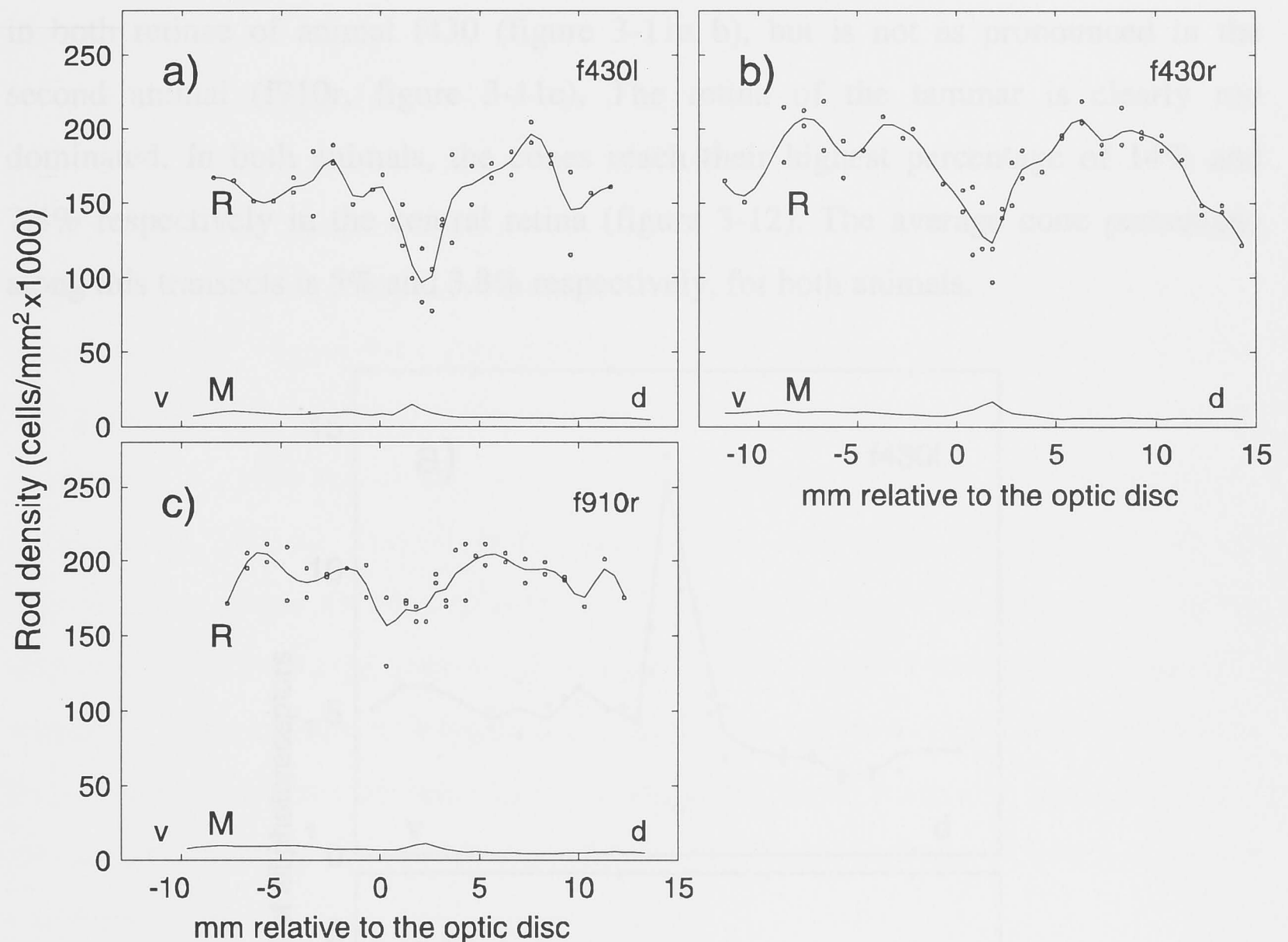


Figure 3-11: Density of rod photoreceptors along ventral-dorsal transect in three retinæ from two animals. 11a, b) are from the left and right retina, respectively, of the same female. Dots show measurements at individual sampling locations and the solid line shows a thin-plate spline function through these data points. The solid line at the bottom of each graph is the cone density in the same retina plotted for comparison. The drop in rod density in 11a, b clearly coincides with the raised cone density in the centre of the retina. Abbreviations: Ventral (v), dorsal (d), rods (R), M-cones (M).

Rods

In two animals we also measured the density of rod photoreceptors along a ventral-dorsal transect (figure 3-11). The rod density stays constant throughout the transect, but drops in the central retina. This drop coincides with the peak in the cone density (solid lines at the bottom of the curve). The drop in density is clearly visible in both retinæ of animal f430l (figure 3-11a, b), but is not as pronounced in the second animal (f910r, figure 3-11c). The retina of the tammar is clearly rod dominated. In both animals, the cones reach their highest percentage of 14% and 7.4% respectively in the central retina (figure 3-12). The average cone percentage along this transects is 5% and 3.8% respectively, for both animals.

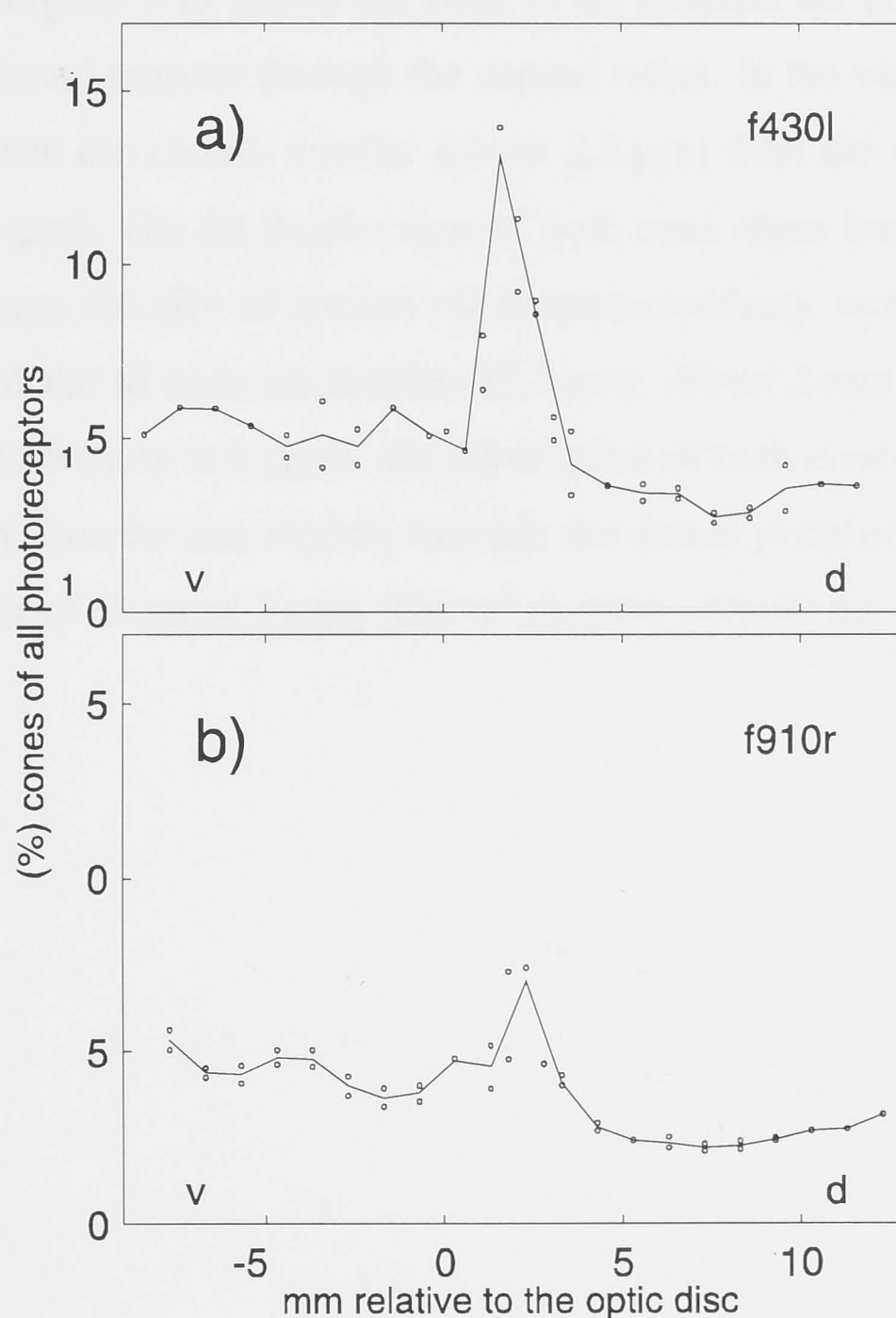


Figure 3-12: Percentage of cones of all photoreceptors along two ventral-dorsal transects for two animals. Dots show percentages at individual sampling locations and the solid line

represents a thin-plate spline function through these data points. Rods clearly dominate throughout the transect. There is only a sharp, thin peak of a slightly higher cone percentage in the central retina. The overall percentage of cone cells along this transect is 5% in 12a and 3.8% in 12b. Abbreviations: Ventral (v), dorsal (d).

Oil droplets

While analysing the whole mounts, we had the impression that the size of the cone outer segments was smaller in the ventral retina than in the dorsal retina. Because it was difficult to measure the size of the outer segments in whole mounts, we decided to measure the diameter of the oil droplets, a measure that was thought to reflect cell size. Figure 3-13 shows the sizes of oil droplets for both M and S-cones along a ventral-dorsal transect through the central retina. In the ventral retina, the oil droplets of S-cones are clearly smaller (about $2.2\text{ }\mu\text{m}$) than the oil droplets of M-cones (about $3.4\text{ }\mu\text{m}$). The oil droplet size of both cone types increases towards the central retina where the size of S-cone oil droplets suddenly increases to ($4.7\text{ }\mu\text{m}$), almost the size of the M-cone oil droplets ($5.3\text{ }\mu\text{m}$). About 2 mm dorsal of the OD, where the M-cone density is highest, the oil droplets of both cone classes reach their largest size. They then decline slightly towards the dorsal periphery where they have the same diameter of about ($4.3\text{ }\mu\text{m}$). The oil droplets of both the M and S-cones are colourless.

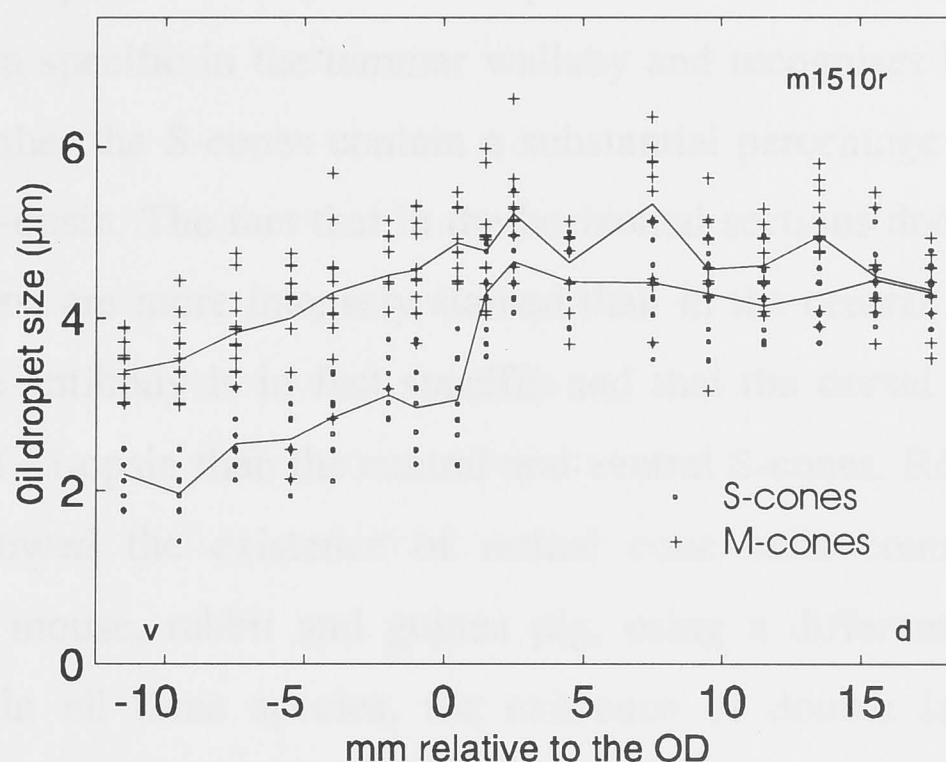


Figure 3-13: Diameter of oil droplets in M and S-cones along a ventral-dorsal transect through the central retina. S-cones were identified by staining with the antibody JH455. Unlabelled cones were taken to be M-cones. For both cone populations the oil droplet size is largest in the central retina. The diameter of the oil droplets in S-cones drops sharply towards the ventral retina where there is a clear difference between the oil droplets of M and S-cones. Abbreviations: Ventral (v), dorsal (d).

Discussion

Antibody specificity

Our results clearly show that there are at least two different cone populations present in the tammar wallaby. The antibody JH455 recognises a small sub-population of cones, whereas JH492 labels the vast majority or all cones in the horizontal sections. In the dorsal retina where the S-cone density is highest, all S-cones are labelled by both antibodies, whereas in the central and ventral retina the situation is not quite as clear, although most S-cones were still found to be double labelled. S-cones label more weakly with JH492 than do M-cones. In the central and

ventral retina, the label with JH492 was often very weak, approaching the background intensity. It is not clear at this point whether the double label indicates that JH492 is non specific in the tammar wallaby and recognises both the S and the M-opsin, or whether the S-cones contain a substantial percentage of the M-opsin in addition to the S-opsin. The fact that in the horizontal sections double labelled cones in the dorsal retina are more intensely stained than in the central and ventral retina, suggests that the antibody is in fact specific and that the dorsal S-cones contain a higher amount of M-opsin than the central and ventral S-cones. Röhlich et al. (1994) convincingly showed the existence of retinal cone cells containing two visual pigments in the mouse, rabbit and guinea pig, using a different set of antibodies (COS-1, OS2). In all three species, the existence of double labelled cones was restricted to the transition zone, which is the area between a field of high M-cone density and a field of high S-cone density. Röhlich and Szél (1993) also showed that the binding sites of their two antibodies are located at the C-terminus side of the respective visual pigments. Using the antibody JH492 in the mouse, Glosmann & Ahnelt (1998), found double labelled S-cones not just in the transition zone, but throughout the ventral retina, indicating that JH492 is either more sensitive than the COS-1 or not specific. On the other hand, in macaques one clearly finds unlabelled cones with JH492 (Martin & Grünert, in prep.) and Leo Peichl (pers. comm.) has found in a range of eutherian mammals, that the S-cones also label with the antibody JH492, but only in restricted areas of the retinae. The fact that the S-cones only double label in restricted areas in these animals, is again evidence for its selectivity. Otherwise one needs to postulate, that the opsin itself or access to the opsin changes between different parts of the retina. There is a clear need to investigate this further, either by showing that the antibody is in fact selective, or more importantly, by recording the spectral sensitivity of individual cone cells.

Spectral sensitivity

In the tammar wallaby, M-cones make up the majority of the cone population. This observation correlates well with results from electroretinogram recordings (ERG) which indicated that the more abundant cone pigment has a peak spectral

sensitivity of about 539 nm (Hemmi et al. submitted). The spectral sensitivity of the S-cones is not quite as clear, as the ERG experiments did not produce any evidence for the presence of a short wavelength sensitive cone population (Hemmi et al. submitted). Behavioural experiments, however, clearly show that the tammar does have dichromatic colour vision and that the S-cone peak sensitivity must be close to 420 nm (Hemmi submitted). The influence of the double labelled cones on colour vision is not clear, but if these cones do in fact contain functional quantities of both opsins, this would presumably decrease the signal to noise ratio in the chromatic channel, but would not exclude colour vision.

Topography of the photoreceptors

Like most mammalian retinae, the retina of the tammar wallaby is dominated by rods which have a very uniform distribution from the ventral through to the dorsal periphery. Their density only slightly drops in the central retina where the M-cone density is highest. Along this transect the overall percentage of cones is only about 3-5% of all photoreceptors.

The topographies of the two cone populations differ markedly from each other and from the rod distribution. The M-cone distribution is most similar to the ganglion cell distribution (Tancred 1981; Wong et al. 1986). There is a sharp horizontal streak of high M-cone density through the central retina which falls off both towards the dorsal and the ventral retina. Unlike the ganglion cells, however, the M-cone distribution shows a strong asymmetry between the dorsal and the ventral retina. After an initial decline from the streak the M-cone density increases again towards the ventral periphery (figure 3-5), whereas it continues to drop towards the dorsal periphery. Also, unlike the ganglion cells which form a clear area centralis in the mid-temporal region on the visual streak, the density distribution of M-cones along the visual streak is very flat and peaks in the central to nasal retina (figure 3-6). The S-cone distribution on the other hand, bears no resemblance to the ganglion cell topography. There are few S-cones in the ventral part of the retina, while they reach their highest densities in the dorsal retina.

The percentage of S-cones is very low (4-6%) throughout the ventral retina. This means that on the ventral side of the visual streak the S-cones follow the M-cone density and match their sharp rise towards the visual streak. On the dorsal side of the visual streak, however, the S-cone density continues to increase towards the dorsal periphery where they reach an unusually high percentage of 30% of all cone cells.

Szél et al. (1996) had concluded that the high density S-cone fields in the rabbit, some mice and the guinea pig are rudimentary areas conserving an ancestral stage of photopigment evolution. We do not think that this interpretation can be extended to the tammar wallaby. In contrast to the examples presented by Szél et al. (1996), where the highest S-cone densities were always found in the ventral retina, the S-cone density in the wallaby is highest in the dorsal retina and the area is not devoid of M-cones which still make up the majority of all cones. We suggest that based on the photoreceptor topography in the tammar wallaby, the retina can be divided into three functionally significant regions: The first region is the dorsal retina, the ventral visual field which has a low ganglion and low cone cell density, but a relatively balanced ratio of M to S-cones and therefore provides the basis for a high sensitivity colour system which is optimised to distinguish objects with different shades of green, such as grasses and herbs. Since the spatial acuity is limited by the ganglion cell density, it would be low. The second region is composed of the horizontal band of high ganglion cell and high cone density across the central retina. It provides for high acuity spatial vision and has the lowest convergence factor between cone cells and ganglion cells. The third region, the ventral retina, has a low density of ganglion cells, but a high cone cell density. The cone density is, depending on eccentricity, about 2-3 times the dorsal density. Also, in contrast to the dorsal retina, the M-cone density is about 20 times higher than the S-cone density. This arrangement would provide poor spectral sensitivity when compared to the dorsal retina and poor resolution compared to the central retina. However, the high convergence ratio between cones and ganglion cells would increase the sensitivity of individual ganglion cells in the dorsal visual field where approaching aerial predators need to be detected. This increased sensitivity may be part of a sensitive early warning system.

Acknowledgements

Oil droplets and double cones

In contrast to the South American opossum where some S-cones and the smaller members of double cones do not contain oil droplets (Ahnelt et al. 1995), all cones of the tammar wallaby contain an oil droplet, including both members of double cones. The oil droplets of both cone classes are smaller ventrally than dorsally. This difference is much more pronounced in the S-cones. Moreover, the size of the S-cone oil droplets increases dramatically just where the pigmentation of the eye suddenly changes from the dark pigmentation of the ventral retina to the light pigmentation of the dorsal retina. These facts agree with the hypothesis that oil droplets alter the light sensitivity of cones by acting as lenses to collect light (Young and Martin 1984). There is more light available in the ventral retina. The oil droplets could potentially act as UV filters to protect the cone outer segments, although the cornea and the lens could perform this task more effectively.

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From spectral sensitivity and its distribution to true colour vision

The results of the immunocytochemical study (chapter 3) clearly showed that the retina of the tammar wallaby contains at least two different cone photoreceptors. The two populations have very distinct distributions across the retina. Together with the ganglion cell distribution, they can be interpreted as dividing the retina into three functional regions, organised as horizontal bands. The ventral retina, which basically looks into the sky contains a large number of cones but few ganglion cells. Therefore, while spatial acuity in this region would be low, the sensitivity of individual ganglion cells would be high, which could provide a sensitive warning system for approaching aerial predators. The central band of the retina is specialised for visual acuity. It is formed by the visual streak with its high ganglion cell density, and has the highest cone density. The cones are mostly M-cones as in the ventral retina. Finally, in the dorsal retina, the densities of both ganglion cells and M-cones are low, but that of S-cones is relatively high. In this part of the retina they reach an unusually high percentage for mammals (30%). This region should therefore be able to provide a sensitive colour signal, optimised to distinguish colours in the blue-green range, which may be useful to distinguish different herbs, or assess the freshness of foliage.

The results reported in chapter 3 also suggest that the failure to find S-cones in the electrophysiological study was due to low signal to noise ratio based on a low overall S-cone density in the tammar retina. There is also the possibility that most S-cones contain some M-cone pigment. If this were the case, it would adequately explain the electrophysiological results.

At this point a behavioural study was clearly needed in order to assess the role of the S-cone population in the function of the visual system. In chapter 4 results of such a study are presented, which show that the tammar wallaby has indeed colour vision, as defined by the ability to discriminate lights based on their hue, independent of intensity. The results also clearly show the presence of a functional S-cone population and measure its peak spectral sensitivity to be about 420 nm.

Chapter 4

Abstract

Dual alternative forced choice experiments with seven stimuli of different intensities were conducted in order to determine the colour vision of the tammar wallaby (*Macropus eugenii*). The wallaby clearly distinguished seven stimuli of different intensities.

Behavioural colour vision in the tammar wallaby (*Macropus eugenii*) and the spectral sensitivity of its S-cone

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Introduction

Despite earlier assertions that most mammals are colour blind, colour vision has in recent years been demonstrated in a variety of eutherian mammals from a wide

Abstract

Dual alternative forced choice experiments, with monochromatic lights as the stimuli were conducted in order to describe the colour vision of the tammar wallaby (*Macropus eugenii*). The wallabies clearly have colour vision, as they are able to discriminate between different colours based on their hue, regardless of their intensity. From the range of monochromatic colours which the wallabies can discriminate and from the fact that a Null-point could be demonstrated, it can be concluded that their colour vision is dichromatic in nature. From the precise location of this Null-point, the spectral composition of the stimulating lights and the known spectral sensitivity of its middle wavelength sensitive cone pigment (539 nm), it is possible to calculate the peak spectral sensitivity of the wallaby's short wavelength pigment to be at about 420 nm. The tammar's colour vision, with the ability to discriminate colours just 3 nm apart, is quite acute. The experiments also suggest that when the discrimination of two colours is impossible based on the wallaby's two cone pigments, the animals are, under these experimental conditions, able to use rod-cone interactions to discriminate these same colours. The wallaby shows a typical mammalian complement of short and middle wavelength sensitive cone photoreceptors, reinforcing the view that the earliest mammals had dichromatic colour vision.

Introduction

Despite earlier assertions that most mammals are colour blind, colour vision has in recent years been demonstrated in a variety of eutherian mammals from a wide range of different orders (see Jacobs 1993 for a review). In marsupials, however, the situation is quite different. The only available information comes from an early study in the Virginia opossum (*Didelphis virginiana*) which showed that they are able to make some colour discriminations (Friedman 1967). The study did not characterise, however, the nature of this colour vision in respect to how many pigments are involved and what the spectral sensitivities of these pigments are. Surprisingly, there is a complete lack of information in regards to colour vision in the Australian marsupials. These animals are a highly evolved and diverse group of mammals which offer a rich source of information, especially from a comparative perspective. There has been quite some interest in their visual systems, with numerous studies carried out with respect to function, development and retinal topography, but not colour. Part of the reason for the lack of data, with regard to spectral sensitivity and colour vision, is most likely their reputation of being rather difficult subjects for behavioural experiments and their (often assumed) status as crepuscular or nocturnal mammals.

Recent immunocytochemical evidence showed that the retina of the tammar wallaby contains at least two different cone classes that coexist throughout the retina (Hemmi and Grünert submitted). Most of the cones belong to the middle wavelength sensitive cone class (M-cones). The percentage of the putative short wavelength sensitive cone (S-cone) is low throughout the ventral retina (5% of all cones), but in the dorsal retina or ventral visual field, the S-cone percentage (30%) is unusually high for a mammal. The wallaby therefore seems to have the potential for quite acute colour vision in this part of its visual field, despite the fact that the overall cone population is relatively small compared to that of the rods (3-5%). In contrast, an attempt to measure the spectral sensitivity of the wallabies' photoreceptors showed only evidence for one M-cone class with a peak sensitivity at 539 nm and rods with a peak sensitivity at about 500 nm. No evidence was found through these measurements to indicate the presence of S-cones at all (Hemmi et al. submitted). Similar results with respect to the anatomy (S-cones present: *Monodelphis domestica*,

Wikler and Rakic 1990; *Didelphis marsupialis aurita*, Ahnelt et al. 1995) and physiology (no evidence for S-cones: *Didelphis virginiana*, Jacobs 1993) have been reported in the American opossums.

With this discrepancy between the anatomical and physiological results in mind, a behavioural study of colour vision in the tammar wallaby was undertaken. The tammar is a kangaroo like member of the order of the Diprotodontia, weighing between 4-8 kg. It can be found in southern Australia where it spends most of the day hidden in the scrub, increasing its activity later in the afternoon and leaving the scrub to feed in the open grass plains after dusk (Inns 1980). Therefore, even though these wallabies forage throughout the night, they are active for a significant amount of time during photopic light conditions (Blakers 1972).

The experiments reported here are designed to show that the tammar wallaby has colour vision, to describe the range over which it can discriminate between monochromatic lights and to estimate the number and the spectral sensitivity of the visual pigments involved in these discriminations.

Materials and Methods

Animals

Two adult tammar wallabies (*Macropus eugenii*) were used in this study, one male and one female. Both animals were born in a breeding colony, kept for experimental purposes, where all animals are kept in small social groups in outdoor paddocks. During the period of actual testing, each animal was separated from its group, by dividing the group's paddock in two. All testing occurred in the animals' outdoor home paddocks and therefore in their familiar environment.

Apparatus

The visual stimuli consisted of two transparent panels separated by 10 cm, onto which coloured light, 50 mm in diameter, could be projected from the back. The lights were produced by two halogen projector lamps (Osram 150 W, 10 V or 20 V). The colour and the intensity of the two lights could be varied independently by

moving Neutral density and interference filters into the light paths. Interference filters were available at approximately 10 nm intervals except for 530 nm (420, 433, 439, 453, 460, 473, 479, 489, 500, 512, 523, 538, 552, 560, 571, 579, 590, 600, 615, 620, 630, 639). All filters were narrow band-pass filters with a spectral half energy bandwidth of less than 20 nm (most: 12-14 nm). All filters, including neutral density filters, were measured with two calibrated spectrophotometers (s1000, Ocean Optics Inc.; casi, ITRES instruments Inc.) for their spectral response properties. All intensity and response calculations are based on the actual measured spectral properties for each filter. The filters were mounted on 9 filter wheels inside the box and could be moved in either one or neither of the two light paths. Up to 8 different intensity matched colours on two intensity levels could be presented in this way in a randomly interleaved fashion, or fewer colours on more intensity levels. The wallabies indicated their choice for a particular colour by pressing the panel onto which the colour was projected. The stimulus presentation, the recording of the responses, the storing of the data and the delivery of the food rewards were all computer controlled and did not require the experimenter's presence. The entire apparatus was housed in a box (64 x 39 x 57 cm, h, w, d), which was placed in the animal's outdoor paddock. The food reward consisted of one pellet of their normal supplement food.

Design

All experiments presented in this paper are based on a dual forced choice design with a correction procedure. That is, two different colour stimuli were presented at the same time, one of them associated with the reward. If the animals made the correct choice, the machine beeped and immediately delivered a small food reward. After a wrong choice, the exact same stimulus condition was presented again until the animals chose correctly. Thus, during these correction trials the animals only needed to change side and choose the opposite panel to make a correct choice. After each wrong response the lights were turned out and the apparatus remained unresponsive for 10 seconds. Following this time-out the lights came back on and the wallabies were allowed to make a new choice. Only the first choice for each stimulus configuration was counted. The correspondence between filters and filter wheels was

randomised daily in order to prevent any consistent correlation of noises produced by the moving wheels with the correct stimulus. Frequent controls performed throughout the experiments showed consistently that the animals based their choices on the actual visual stimulus presented on the panels.

Because no spectral luminosity function was available for the wallabies, the intensities of different colours were equated for the wallaby eye by assuming that the perceived intensity is based on a ratio of 6 M-cones to 1 S-cone. M-cones are known to have a peak sensitivity of 539 nm (Hemmi et al. submitted), whereas for the S-cones a peak sensitivity at 430 nm was assumed, since this is common in other mammals. The ratio of M and S-cones used lies in between the empirically measured dorsal and ventral ratios of cones in the tammar wallaby (Hemmi and Grünert submitted). Because this procedure cannot guarantee a perfect intensity match, the intensity of the stimuli was constantly varied in all experiments to mask potential intensity miss-matches and make it impossible for the animals to use intensity differences between the lights to make the required discriminations. Changing the intensity ratio between the two presented stimuli also allowed the results to be inspected for potential intensity influences.

Procedure

Individual animals were first trained to associate the pressing of the panels with food and were then introduced to visual discriminations, either by training them to discriminate light from no light (female, f037) or directly green from blue (male, m400). Testing was consistently performed overnight, starting at dusk and stopping at dawn. There was no limitation on the number of trials and the animals could work according to their own motivation during their main activity period (Blakers 1972). Apart from grass which was available in the wallabies' paddocks, the animals were not fed any additional food. The experiments easily provided enough food during a night. In fact on most mornings, a substantial pile of food rewards was found uneaten, indicating, the animal's had continued to work beyond their hunger based motivation. For each experiment, the final data collection was started, after the animals reached a stable response pattern. Statistical results are based on the binomial distribution.

Experiment 1: Colour discrimination

The animals were trained to choose between a blue light (453 nm) and a green light (550 nm). For one animal (f037), the blue light was the positive stimulus and for the other animal (m400), the green light was the positive stimulus. The intensity of both lights was varied in four steps from 120 to 0 (black) cd/m^2 for the blue light and 500 to 0 (black) cd/m^2 for the green light. The experimental design was based on blocks of 50 trials, including an equal number of presentations for all 25 possible intensity combinations and an equal number of left or right presentations for the positive stimulus for all 25 combinations. Presentation of trials within each block was fully randomised. Several blocks were presented per night. Unfinished blocks of trials were included in the final analysis. This design made it impossible for the wallabies to use intensity as a cue to distinguish the two colours consistently.

Experiment 2: Range of colour discriminations

In order to measure the range of wavelengths over which the wallabies are able to discriminate colours, the wallabies were trained for a relative colour discrimination task. The animals had to select, from any given pair of monochromatic colours, the colour with the longer wavelength, a task they performed easily. If for instance 480 nm was presented against 500 nm, the animal was trained to choose 500 nm. But if 500 nm was presented vs 520 nm, the animals had to choose 520 nm and so on. The colours presented were always 20 nm apart and ranged from 440 nm to 570 nm (f037) or 420 nm to 570 nm (m400). This design meant that the wallabies did not have to be retrained for every new colour that was introduced. They readily transferred the concept between colour pairs. Because of the uncertainty about the exact intensity match between the two different colours, they were always presented in such a way that either one of the two colours (either the positive or the negative colour) was reduced in intensity by a factor of three, by moving a neutral density filter into the light path. The design was again based on blocks of equal numbers of trials for each colour combination, equal numbers of trials during which the positive or the negative colour was reduced in intensity and equal numbers of left or right positive trials. The

presentation of trials within each block was again fully randomised and unfinished blocks of trials were included in the analysis. The intensity of the bright lights in this experiment and all the following experiments was the equivalent of 570 cd/m^2 as measured for a 530 nm light.

Experiment 3: Null-point

The wallabies were kept on the exact same training and were rewarded as in experiment 2, except that only colours in the range of 460-520 nm were used. Then, on every sixth trial, instead of presenting a relative colour combination, one colour was replaced by a white stimulus of the same intensity as the colours. The animal's responses towards these 'white' trials were recorded, but the animal was not rewarded, regardless of its choice. Instead, a new training trial started immediately.

If the tammar wallaby is a dichromat, white will have the subjective appearance of a monochromatic colour of a particular wavelength or hue (Neumeyer 1991). The idea is that for a given pair of photoreceptors, one can always find a monochromatic colour that excites the two photoreceptors in exactly the same way as a white stimulus of a defined spectral composition. If such a wavelength or Null-point can be found in the wallaby, the animals should readily order the white into the range of presented colours according to the white's subjective appearance. Trained to choose the longer wavelength, the wallabies should prefer white over short wavelength colours but should prefer long wavelength lights over white. The exact point where the wallaby places the white on the wavelength axis, corresponds to the Null-point. No such point exists for trichromats.

This experiment is essentially a colour mixing experiment. The expected position of the Null-point depends only on the spectral response of the photoreceptors, the spectral characteristics of the eyes' optics and the spectral composition of the stimulating lights. Under moderate intensity conditions (i.e. no saturation) the location of the Null-point does not depend on intensity. It also does not depend on any photoreceptor characteristics that are not wavelength dependent, including the subsequent weighting of the photoreceptor responses by the visual system. The optics of the wallaby's eyes and the oil droplets of their cones (Hemmi

and Grünert submitted) look colourless to a human eye. Also, measurements of the rod spectral sensitivity in the intact eye (electroretinogram) and in isolated cone cells (microspectrophotometry) gave almost identical results (Hemmi et al. submitted). This indicates that there is not significant differential absorption by the optics for the visible light. Whether the wallaby's optics and the oil droplets of their cones transmit UV light is not known. However, because the light from halogen light bulbs does not contain large amounts of UV, the results do not depend on the high pass characteristics of the optics, as long as they transmit light from 390 nm onwards (even for a cut-off at 400 nm the results change only by a couple of nanometers). All cone spectral sensitivity functions (nomograms) were derived from Stavenga et al.'s (1993) exponential functions using the appropriate wavelength for the peak sensitivity.

Three controls were run in order to show that the wallabies responses during the test trials were indeed a relative colour discrimination task between the white and the colours and that the position of the measured Null-point changes according to changes in the spectral composition of the white light, as predicted by the above calculations. The controls were run under the exact same experimental conditions as outlined above, except that the white was replaced by a light with different spectral characteristics: a) A 560 nm monochromatic light was used. Trained for the relative discrimination task, the wallaby should always prefer the 560 nm light; b) The white light was filtered by a 450 nm long pass cut-off filter, which should act to shift the measured Null-point towards longer wavelengths; c) The white light was filtered by a 550 nm short pass cut-off filter, which should have the effect to shift the Null-point towards shorter wavelengths. The exact distance for the shifts in b and c, can be calculated once the Null-point for the basic white has been determined (see below). The intensities of all these lights were matched to the monochromatic colours.

Experiment 4: Colour discrimination in the red

The wallabies were trained to discriminate a long wavelength light of 640 nm from a range of monochromatic lights. The animals were rewarded for choosing the 640 nm light. The left and right presentations of the positive stimulus and the

intensities of the presented colours were randomised as in experiment 2. For one animal (f037), the intensity difference between the two presented colours was increased to a factor of 50. The reliability of the discriminations of different monochromatic lights from 640 nm was expected to match the sensitivity of the long wavelength slope of the S-cone. We also repeated experiment 2 with one animal, but this time the difference between the presented colours was 30 nm and the range over which testing occurred was 420-640 nm.

Results

General performance

After having learned to operate the apparatus, both animals readily learned to discriminate between the presented colour stimuli. The female, f037, first learned a brightness task. After she performed this discrimination reliably, she learned in the course of one night to discriminate a green from a blue light. The male, m400, which was immediately confronted with a colour discrimination task after having mastered the basic apparatus took slightly longer but performed at a 70% correct choice level during the third night of training. The order in which the results are being reported is the order in which m400 performed the experiments. The corresponding order for f037 would be: 1 4 2 3.

Experiment 1: Colour discrimination

The first experiment shows that the wallabies can make colour discriminations between a 453 and a 550 nm light independently of light intensity. Figure 4-1 shows the percentage of correct responses for the 25 intensity combinations plotted against the intensity of the negative colour. The results are based on a minimum of 47 trials per data point. The intensity of the positive stimulus is encoded in the numerals 0-4 in order of increasing light intensity. Both animals chose the positive colour more often when it was presented against black, but chose black more often when it was presented against the negative colour, even though these conditions were presented in

a random order. This means that they learned both the positive and the negative colour. In fact, m400 was able to discriminate the two lights under all intensity conditions except when both lights were black (point 0/0), which was a control condition for experimental artefacts. F037 also failed to distinguish the two colours under the control condition, but also failed to discriminate between a very weak negative stimulus and black. This does not contradict the conclusion, however, that the wallabies distinguish the two colours based on hue rather than intensity, but shows that she found it difficult to respond to a weak negative stimulus in the absence of a positive stimulus.

Experiment 2: Range of colour discriminations

In order to determine the range of wavelength over which the wallabies can discriminate between monochromatic stimuli, the animals were trained to perform a relative colour discrimination task. That is, when presented with two monochromatic colours 20 nm apart, they had to choose the colour with the longer wavelength, regardless of what particular colours were presented. Both animals learned this task very easily. It is interesting to note that the male (m400) who in the previous experiment (453 vs 550 nm) was trained to choose 550 nm, chose the longer wavelength colours from the start, suggesting that to him both tasks involved the same choice criteria.

Figure 4-2 shows the percentage of correct choices plotted against the mean wavelength of the two presented colours. The range of wavelength the wallabies can distinguish under these conditions is limited to between 440 and 530 nm (black symbols: $p \leq 0.01$; grey symbols: $p \leq 0.05$; open symbols: ns). For both animals the curve shows a single peak in the blue-green region, at 470 nm (m400) or 480 nm (f037) and drops of to the 50% level towards shorter and longer wavelengths.

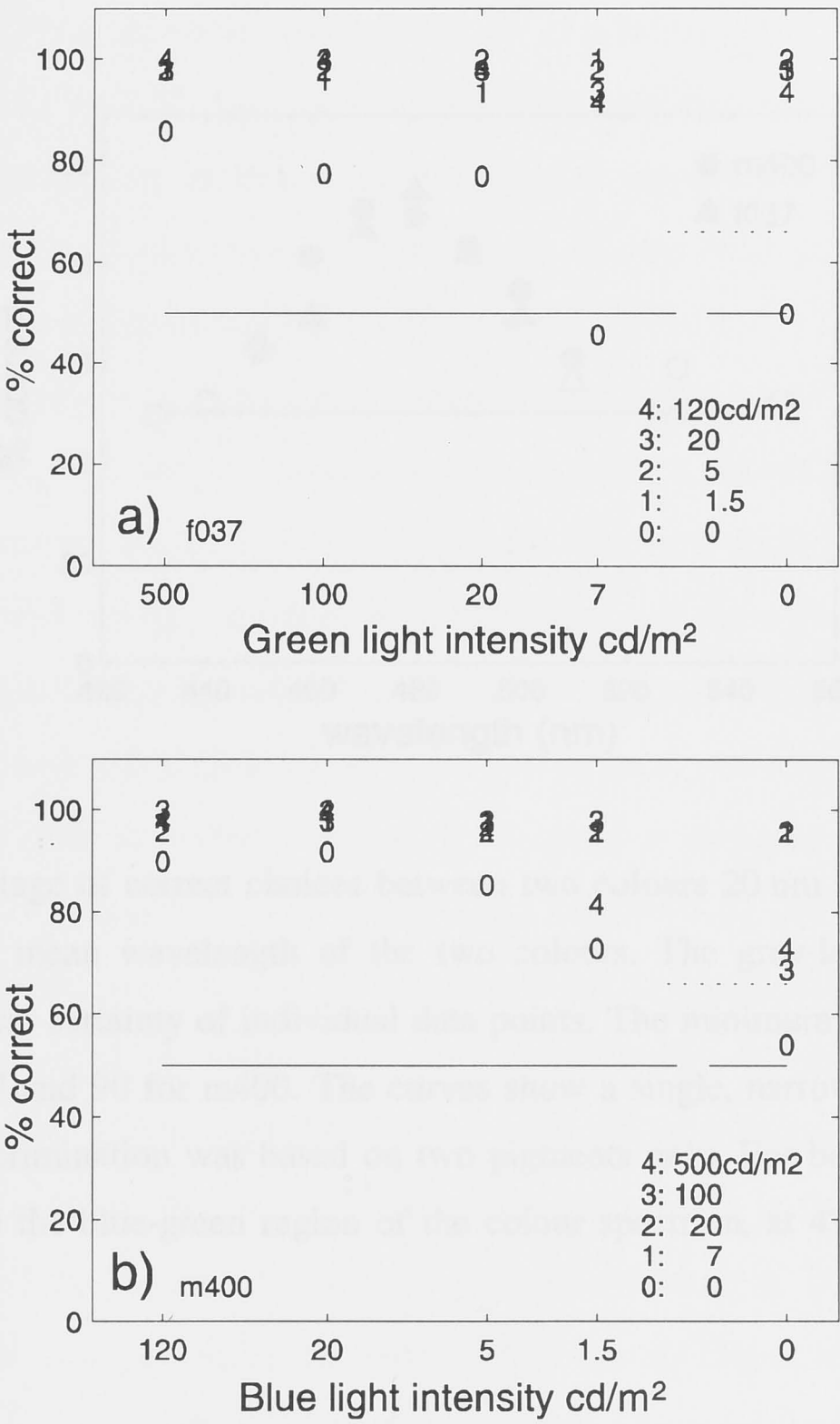


Figure 4-1: Discrimination of two monochromatic lights under a wide range of intensity combinations. Each point represents the percentage of correct discriminations based on a minimum of 47 repetitions. The dotted line indicates a significance threshold of $p < 0.05$. a) The results for m400. The intensity of the negative light (blue, 453 nm) is plotted along the x-axis and the intensity of the positive light (green, 550 nm) is encoded in the numerals 0-4 in increasing intensity. b) The results for f037. In this case blue was positive and green negative. The discrimination between the two colours is clearly based on hue and not intensity.

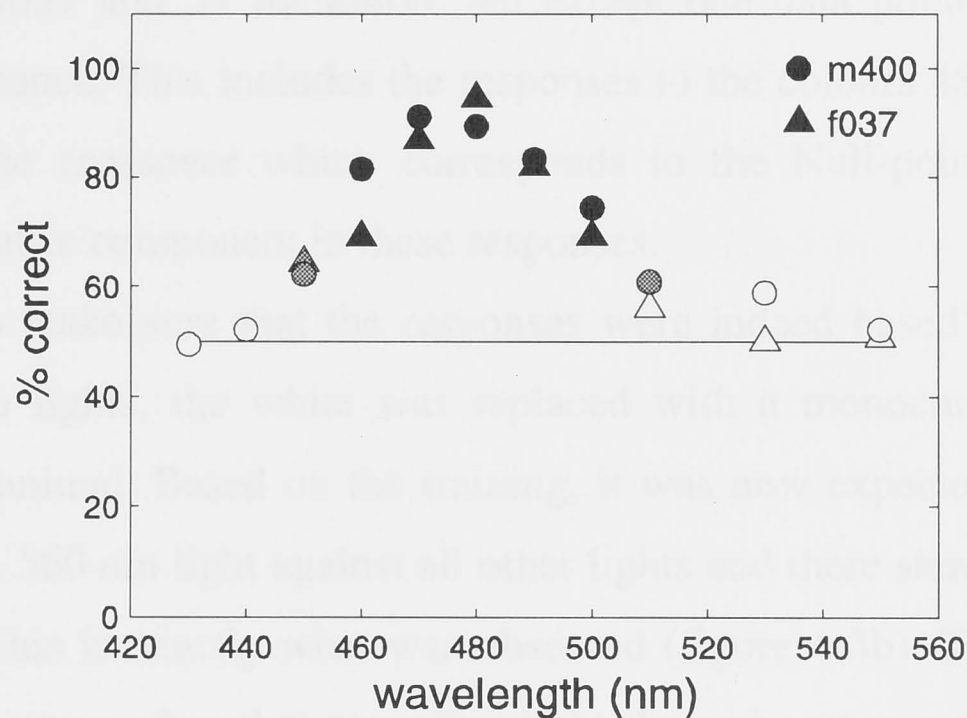


Figure 4-2: Percentage of correct choices between two colours 20 nm apart. Responses are plotted against the mean wavelength of the two colours. The grey-level of the symbols indicate the statistical certainty of individual data points. The minimum number of trials per point is 52 for f037 and 90 for m400. The curves show a single, narrow peak, as would be expected if the discrimination was based on two pigments only. For both animals the peak performance lies in the blue-green region of the colour spectrum, at 470 nm for m400 and 480 nm for f037.

Experiment 3: Null-point

The relative colour task can also be used to determine whether the wallaby is a dichromat and measure the location of the Null-point. The set of presented colours was limited to between 460 and 520 nm and the exact same training protocol was used as in the previous experiment. But now, every sixth trial was replaced by an unrewarded test trial during which the animal had to choose between one of the monochromatic colours and white. The results are very clear and are shown in figure 4-3a for both animals. When white was presented together with a short wavelength colour (460-480 nm), both wallabies chose the white panel more often, thus treating white as if it had a hue corresponding to a longer wavelength than 480 nm. On the other hand, when white was presented against the longer wavelength (490-520 nm),

the wallabies chose the monochromatic colours. The minimum trial number per data point is 21 for f037 and 37 for m400. All except one data point are significantly different from chance. This includes the responses to the colours 480 and 490 nm on either side of the crossover which corresponds to the Null-point. There was no consistent luminance component in these responses.

In order to make sure that the responses were indeed based on a comparison between the two lights, the white was replaced with a monochromatic colour of 560 nm for one animal. Based on the training, it was now expected that the animal should prefer the 560 nm light against all other lights and there should be no reversal in preferences. This is exactly what was observed (figure 4-3b). The wallaby chose the 560 nm light more often than any other light. Indeed, not one mistake was made up to 490 nm. The drop in performance at the longer wavelengths reflects the general drop in performance towards longer wavelengths (figure 4-2) and the decrease in the difference between the two colours. Results are based on 10-12 trials per point.

In a second control, the white light was filtered with a 450 nm long pass cut-off filter (Orion SS-450), which appears slightly greenish to a human. From theoretical calculations (see methods) one would expect that this change in the white light would shift the measured Null-point towards a longer wavelength by about 15-17 nm, depending on the exact spectral sensitivity of the S-cone. The results shown in figure 4-3c are based on a minimum of 27 trials per point. The results are consistent with a shift in the Null-point to just above 500 nm. The wallaby clearly preferred the long pass filtered white light against colours from 460-490 nm, but failed to discriminate it from the longer wavelengths. By long pass filtering the white light not only did the Null-point shift towards higher wavelengths, but also away from the point of highest sensitivity for wavelength discriminations which in the wallaby lies between 470 and 480 nm (figure 4-2). It is therefore not surprising that no preference reversal takes place and the wallaby simply failed to make any discriminations above 500 nm.

In an attempt to localise the position of the Null-point more accurately, the white light was also filtered with a 550 nm short pass cut-off filter (Orion LS-550), which changed the white to appear slightly bluish to humans. The calculated shift of the Null-point under these conditions was small, in the order of 2-4 nm. The results

are based on a minimum of 25 trials per point (f037) or 29 trials per point (m400) and are shown in figure 4-3d for both animals. The result of the male is essentially the same as seen in figure 4-3a, with the exception that, as might be expected, he found it easier to discriminate the short pass filtered white from the longer wavelength (490-520 nm), but found it more difficult to distinguish 480 nm from the short pass filtered white than from the original white. The position of the measured Null-point was still between 480 and 490 nm. The responses of the female (f037) showed the same trend, but were less accurate in the critical region, which is probably a reflection of her motivation rather than ability to discriminate the colours (she gave birth during the experiment and essentially stopped working after a few days).

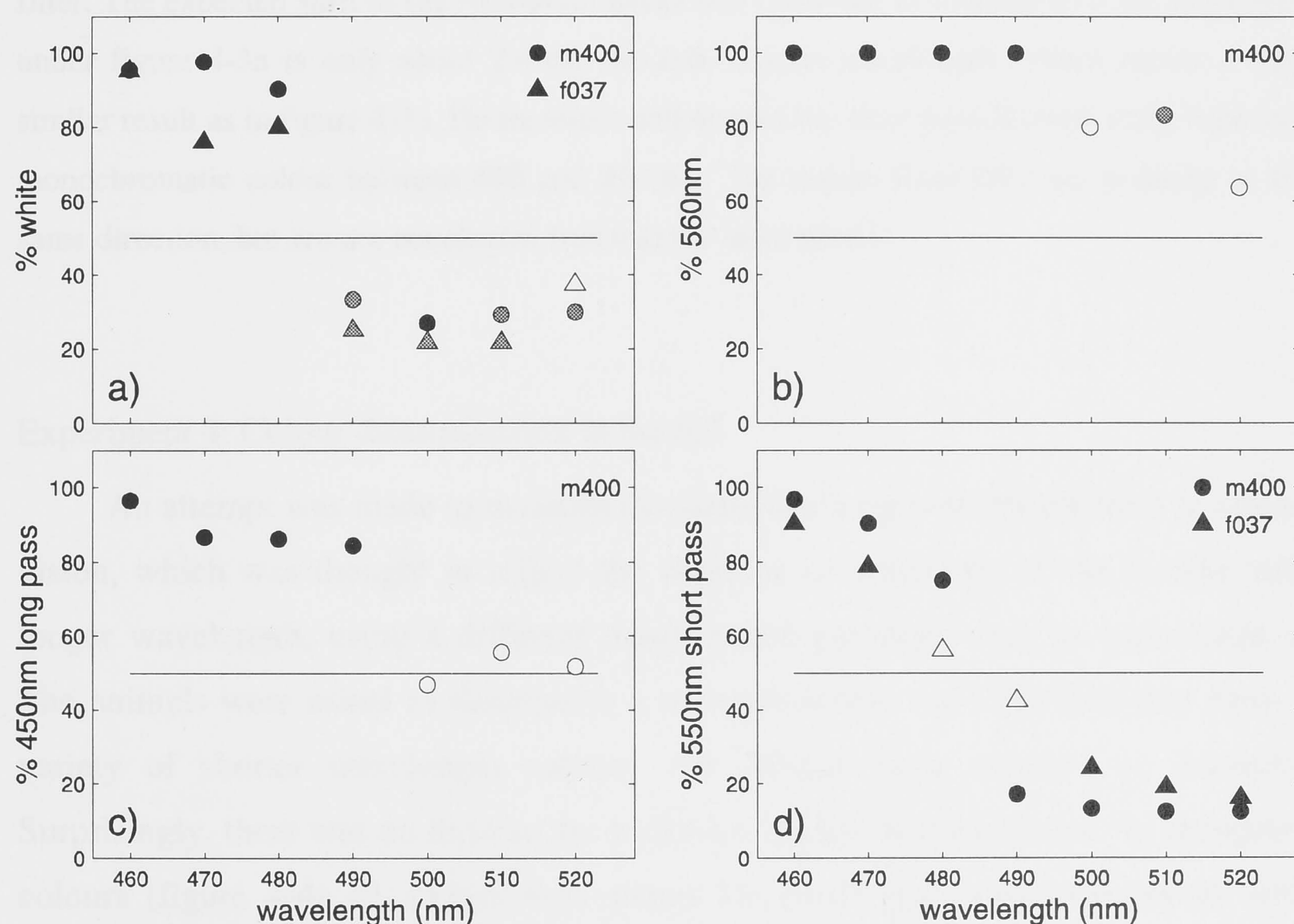


Figure 4-3: Determination of the Null-point in the tammar wallaby. The tammars were trained to choose the colour with the longer wavelength as in experiment 2 and were then asked in unrewarded trials to discriminate between monochromatic colours and white lights of different spectral compositions. The percentage of choices of the white stimulus is plotted against the wavelength of the monochromatic colour (black symbols: $p \leq 0.01$; grey symbols:

$p \leq 0.05$; open symbols: ns). a) Results from trials using the unfiltered white light. Both wallabies prefer white against the shorter wavelength colours (460-470 nm) but prefer the longer wavelength colours (490-520 nm) over white. They, therefore, treated white as having the hue of a monochromatic colour with a wavelength between 480 and 490 nm. b) The white was replaced by a 560 nm monochromatic light. The animal chose the 560 nm light more often than any other light. The 490 nm light which was avoided when presented against white in the previous experiment has now been chosen in 12 out of 12 cases. c) The white light was filtered by a 450 nm long pass cut-off filter. The expected shift of the Null-point under this condition is about 15-17 nm towards a longer wavelength. There is no clear reversal of preference in the data. Instead, the animal (m400) preferred the long pass filtered white against the shorter wavelength, including 490 nm, but fails to distinguish the shifted white from the longer wavelength. d) The white light was filtered by a 550 nm short pass cut-off filter. The expected shift of the Null-point under this condition as compared to the conditions under figure 4-3a is only about 2-4 nm towards shorter wavelength. M400 shows a very similar result as in figure 4-3a. He therefore still treated the short pass filtered white light as a monochromatic colour between 480 and 490 nm. The results from f037 are pointing in the same direction, but are not conclusive (see text for more detail).

Experiment 4: Colour discrimination in the red

An attempt was made to measure the wallabies' long wavelength limit to colour vision, which was thought to reflect the decrease in sensitivity of the S-cone with longer wavelength, using a different experimental paradigm than in experiment 2. The animals were asked to distinguish a monochromatic red light (640 nm) from a variety of shorter wavelength colours. All colours were matched in intensity. Surprisingly, there was no limit to the wallabies' ability to discriminate the presented colours (figure 4-4a, b). Figure 4-4a shows the result from two experiments with f037. The triangles show the mean response to a variety of different intensity conditions. The intensity of both colours was varied over 2 steps from full intensity (570 cd/m^2 as measured for the 530 nm light) to about 10 cd/m^2 and all 9 possible colour combinations were presented. In the second experiment, in any given trial either one of the two colours (positive or negative) was presented darkened as in

experiments 2 and 3. The difference in intensity between the lights was large, a factor of 57. The circles represent the mean response and the line associated with each circle shows the difference between the two intensity conditions.

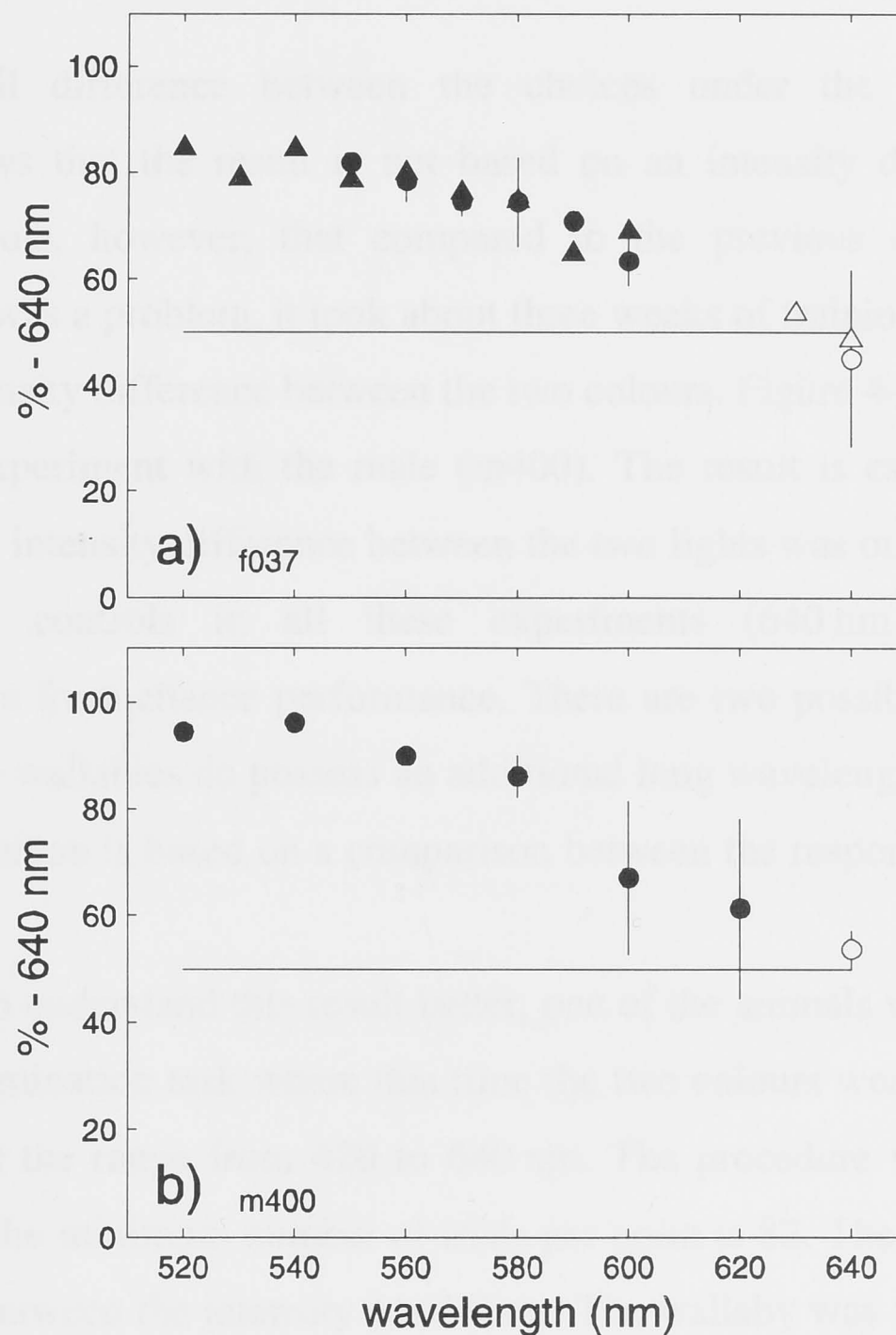


Figure 4-4: Results of an experiment where the animals were trained to discriminate between a 640 nm light and various lights of shorter wavelength. Percentages of choices for 640 nm are plotted against the wavelength of the negative colour (black symbols: $p \leq 0.01$; open symbols: ns). The minimum number of trials for any point is 128 for figure a and 177 for figure b. a) The results of two experiments with f037. Triangles show the mean response for an experiment where the intensity of both colours was varied in two steps over more than a factor of 50 from 570 to 10 cd/m^2 (as measured for the 530 nm light). All 9 intensity combinations were presented. For the second experiment only the two most extreme conditions were presented (positive dark and negative bright and vice versa). The circles

represent the mean response and the vertical lines show the difference between the two intensity conditions. The small lines clearly show that intensity was being ignored. Note that controls from both experiments (640 nm vs 640 nm) were indistinguishable from 50%. b) The second experiment was also repeated in the other animal m400 with the same result.

The small difference between the choices under the different intensity conditions shows that the result is not based on an intensity discrimination. It is important to note, however, that compared to the previous experiments where intensity never was a problem, it took about three weeks of training before the animal ignored the intensity difference between the two colours. Figure 4-4b shows the result for the same experiment with the male (m400). The result is essentially the same, even though the intensity difference between the two lights was only a factor of three. Note that the controls in all these experiments (640 nm vs 640 nm) are indistinguishable from chance performance. There are two possible explanations for this result a) the wallabies do possess an additional long wavelength cone pigment, or b) the discrimination is based on a comparison between the responses of the rods and the M-cones.

In order to understand this result better, one of the animals was trained again in a relative discrimination task where this time the two colours were 30 nm apart. The colours covered the range from 420 to 640 nm. The procedure was the same as in experiment 2. The minimum number of trials per point is 82. The vertical lines show the difference between the intensity conditions. The wallaby was able to discriminate the lights right up to 615 nm. Choice performance shows a clear peak between 470 and 480 nm, as in experiment 2. Towards the shorter wavelengths, the result drops rapidly to 50%, but towards the longer wavelengths, the percentage of correct choices remained at about 70%. There is no second peak in performance however, as would be expected if the wallabies had a second long wavelength pigment. Also, there is a clear intensity influence visible in the data which has not been found in any of the previous experiments. The animal had a consistent bias towards the darker stimulus. Both intensity conditions, however, are above 50% in the long wavelength region of the spectrum. Therefore, the result cannot be explained by intensity alone. The

differences between the intensity conditions do indicate, though, the increased importance of the luminance channel under these conditions.

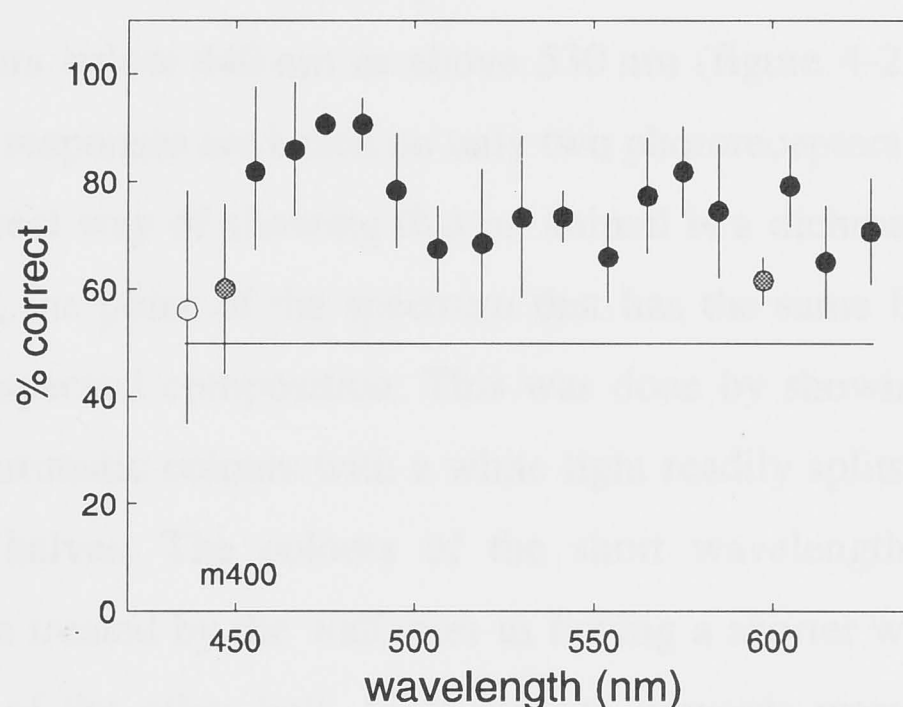


Figure 4-5: Repetition of experiment 2 with a relative difference between the two colours of 30 nm. The circles represent the mean response (black symbols: $p < 0.01$; grey symbols: $p < 0.05$; open symbols, ns) and The vertical lines represent the difference between the two tintensity conditions. The labels are as in figure 4-2. The peak performance is in the same place as for 20 nm, but on the long wavelength side. The results do not reach 50%. Note the strong intensity influence as shown by the long vertical lines.

Discussion

Colour vision

The first experiment showed clearly that the tammar wallaby has colour vision. Both animals consistently discriminated between a green and a blue light across a random presentation of 25 different intensity combinations where both colours were varied from over 100 cd/m^2 to black (0 cd/m^2). Both animals learned the task easily and performed at almost 100% correct for most intensity combinations, suggesting that colour is a salient stimulus.

The second experiment gives the first indication that the tammar is in fact a dichromat. When asked to discriminate between two monochromatic colours 20 nm apart, the animals performed well over only a narrow range of wavelength with a single peak in performance in the range of 470 to 480 nm. The wallabies could not distinguish colours below 440 nm or above 530 nm (figure 4-2). This narrow range suggests that the responses are based on only two photoreceptors.

A more direct way of showing that an animal is a dichromat is to show that it has a Null-point, the point of the spectrum that has the same hue as an achromatic light of a given spectral composition. This was done by showing that a comparison between monochromatic colours with a white light readily splits the wallabies colour space into two halves. The colours of the short wavelength half, from 480 nm downwards, were treated by the wallabies as having a shorter wavelength than white and the colours of the other half, from 490 nm upwards were treated as having a longer wavelength than white. The transition is very sharp. Thus, in this relative discrimination task the animals treated the white as having a hue equal to a monochromatic colour between 480 and 490 nm. In agreement with this interpretation is the fact that the spectral composition of the white light determines the measured location of the Null-point (figures 3a, c, d) as predicted by theoretical calculations. The apparent hue of the white light for instance could be changed to about 500 nm by filtering it with a 450 nm long pass cut-off filter (figure 4-3c).

The reaction of one animal, the female f037, to the white stimulus when it was first introduced, shows clearly that the observed split of the spectrum into two halves by a white light was not forced upon the animals by an elaborate training routine. F037 had been trained to distinguish 640 nm from shorter wavelength colours in experiment 4, straight after the first experiment and thus had not been explicitly trained for a relative colour discrimination task. After completion of experiment 4, it was attempted to train her to discriminate a range of monochromatic colours from a white stimulus by rewarding the choice of the white light. Surprisingly, she completely resisted the training during her first night and chose the colours in an almost identical way as shown in figure 4-3a. Thus, even though she was clearly able to discriminate the monochromatic colours (which she has never seen before) from

white, she avoided the white light when presented against colours of 490 nm or longer wavelength, despite the fact that she was being rewarded to choose white. At the same time she preferred white over bluish colours. The only interpretation I have for this result is that during experiment 4 she learned to choose the light with the longer wavelength and then transferred this training to the new task, treating white as having a hue between 480 and 490 nm, resisting the training to the contrary. When the training was continued in subsequent nights, her responses deteriorated to chance level for all colours and did not show any sign of recovery for several days at which point the experiment was changed to the relative discrimination format (experiments 2-4). After this, the animal's percentage of correct responses immediately increased.

Spectral sensitivity of the short wavelength sensitive cone.

The fact that the reversal between the white and colour preference in the Null-point experiment lies below 500 nm rules out the possibility that an interaction between the rods and the M-cones could be responsible for these results. In fact, the peak of the second pigment involved in this discrimination task has to be well below the Null-point itself. Given that the spectral sensitivity of the M-cones is known (539 nm; Hemmi et al. submitted), one can now calculate the spectral sensitivity of the S-cone by reversing the Null-point calculations as outlined in the methods section. The calculations are based on the fact that the estimated Null-point lies between 480 and 490 nm (the exact filter measurements are: 479 and 489 nm) for both, the original white light (figure 4-3a) and for the short pass filtered white light (figure 4-3d) and provide enough chromatic contrast against these lights to make a discrimination possible. From this an estimate for the S-cone peak sensitivity of 419 nm follows. The corresponding Null-points are 486 nm for the original white light and 482 nm for the short pass filtered white light. This gives the animal at least 3 nm to distinguish each of the white lights from each of the two monochromatic lights (479 and 489 nm).

From the positions of the calculated Null-points, one can also infer that the wallabies colour vision is quite acute with a minimum $\Delta\lambda$ of at most 3 nm. Given that under the experimental conditions used here, the Null-point does not exactly coincide

with the area of best discrimination performance, the true value is likely to be slightly smaller. This should, however, be shown directly through colour discrimination experiments with smaller $\Delta\lambda$ than used in the present experiments.

Colour perception of dichromats

The animals readiness to learn a relative colour task requires some more attention. Both animals spontaneously used the relative wavelength difference between monochromatic colours to make discriminations when presented with a new set of stimuli. This may seem surprising to a human trichromat. In fact, during the setting up of the relative colour experiments, the experimenter constantly had to consider which colour has the longer wavelength in order to choose the correct stimulus. For a dichromat, however, all colours naturally line up in one dimension which corresponds to the excitation ratio between the two cones (Neumeyer 1991). The results here suggest that this is also how the wallabies perceive these colours. Their colour perception, therefore, seems to resemble (in some aspects) human luminance perception, where relative differences are more important than absolute intensity. The results presented here give no indication that the Null-point splits the spectrum into two halves in a way that subjects can primarily make colour discriminations between the two halves, as suggested by Jacobs and Deegan II (1994). This is demonstrated in figure 4-2, where the peak performance actually falls just to the left of the Null-point. As can be seen from figure 4-3, the location of the Null-point in the spectrum is entirely dependent upon the spectral composition of the white light. Dichromats have a two-dimensional colour space (hue and intensity) and cannot measure saturation. Therefore, any colour (broadband or not) can be matched exactly by a single monochromatic colour. What corresponds to white in humans is just another hue for a dichromat and does not constitute a special point in the animals colour space.

Colour discrimination in the red

It came as a surprise that the wallabies were able to make colour discriminations in the long wavelength region of the spectrum, which they theoretically should not be able to do with their set of S and M-cones. There are two possible explanations. Either the wallabies have an additional long wavelength sensitive pigment, or they are able to use an interaction between rods and cones to perform these discriminations. The experimental conditions, high intensity lights on a dark background (the experiment was run at night), would certainly have provided ideal conditions for joint contributions of both rods and cones. There are several reasons why I think these responses are indeed based on a rod-cone interaction. a) First of all, the results in experiments 2 and 3, where the test colours were kept between 420 and 560 nm, are clearly consistent with a dichromatic observer; b) The relative colour experiment with 30 nm distance between the lights (figure 4-5) shows a clear, well defined peak where the two cones interact, but does not show a second peak further towards a longer wavelength, which would be expected in the presence of an additional long wavelength pigment; c) There is a clear intensity influence in experiment 4 (figures 5) which was not visible in the previous experiments. The intensity influence cannot explain the discriminations, but indicates the increased importance of the luminance signal; d) Experiment 4 was more difficult for the animals which is reflected in the higher number of trials per point and a much longer training period to reduce intensity influences initially seen in figure 4-4a and a higher variance in the results in figure 4-5.

If one assumes that the S-cone responses are replaced by a rod responses whenever the S-cones are not stimulated, one could easily explain all the above experiments. In agreement with this hypothesis is the ease with which the animals transferred to and from experiment 4 to other tasks. This ease implies that for the wallabies these experiments involved the same choice criteria even though the S-cones were not stimulated in experiment 4.

There have been numerous reports in humans that rod-cone interactions can be used for colour discriminations (e.g. Smith and Pokorny 1977; Nagy 1980; Hess et al. 1989). In addition, Jacobs (1993) convincingly showed that the nocturnal monkey

Aotus is in fact a monochromat and concluded, therefore, that earlier studies (Jacobs 1977), which showed rudimentary colour vision in the *Aotus*, probably measured rod-cone interactions.

Comparative aspects

The tammar wallaby has dichromatic colour vision and the spectral sensitivity of its two cone pigments (S-cones 420 nm; M-cones 540 nm) is similar to those of many eutherian mammals. This supports the emerging picture, based on the cones found in a range of eutherian mammals (Jacobs 1993), that the earliest mammals already had two cone pigments and did not have pure rod retinae as earlier believed (Walls 1942).

It is also interesting to compare the tammar wallaby to the American opossums. Based on the fact that their sensitive flicker photometric technique did not find any evidence for a S-cone in the retina of the Virginia opossum, Jacobs (1993) questioned whether the discriminations found by Friedman (1967) might be based on rod-cone interactions. The same problem with regard to the absence of S-cone responses was experienced in the tammar wallaby (Hemmi et al. submitted), but this study shows that their retina does contain a functional S-cone population, as has been predicted by the immunocytochemical experiments (Hemmi and Grünert submitted).

The failure of the physiological recordings to show any evidence of an S-cone population, probably reflects very low signal levels due to the low overall density of S-cones in this species. The same may indeed be true for the opossums, especially because the presence of an S-cone population has also been predicted by immunocytochemical studies in two species (Wikler and Rakic 1990; Ahnelt et al. 1995). A more detailed behavioural study in the opossum seems certainly warranted. There is also a great variety of Australian marsupials still to be investigated, including fully diurnal animals such as the tree kangaroo.

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Summary and Conclusions

This thesis describes a series of experiments that are concerned with some of the fundamental characteristics of the visual system of the tammar wallaby: Spatial acuity, contrast and spectral sensitivity and colour vision and their relationship to the underlying retinal structure and physiology.

In chapter 1 it was shown with behavioural experiments, that the tammar wallaby has a visual acuity of about 4.8 c/deg, which is slightly lower than that of the cat. This result compares well with the animals ganglion cell distribution, which predicts an upper limit to acuity of about 6 c/deg. The electrophysiological estimate of contrast sensitivity obtained with evoked potentials, however, is probably slightly underestimating the true sensitivity in this animal. The measured acuity limit of 2.8 c/deg, using this method, is slightly lower than that demonstrated behaviourally. The reasons for this discrepancy are probably methodological, related to the basically arbitrary definition of response cut-off in evoked potential measurements. These factors should not, however, have affected the shape of the contrast sensitivity function. A better estimate of the tammar's absolute highest sensitivity can therefore be obtained by shifting the function vertically, to achieve a match between the behavioural and the physiological acuity. If this is done, the wallaby shows a similar absolute sensitivity as has been measured in the cat and the rabbit.

An attempt to measure the spectral sensitivity of the wallaby's photoreceptors, as reported in chapter 2, provided clear evidence for a middle wavelength sensitive cone (M-cone) photoreceptor with a peak spectral sensitivity of about 540 nm, which is a typical mammalian M-cone sensitivity (Jacobs 1993). There was no indication, however, that the wallaby has a short wavelength sensitive cone (S-cone). The rod spectral peak sensitivity was measured with both an electroretinogram based method, which records the functional sensitivity in the intact eye and will therefore be affected by the spectral characteristics of the optics and microspectrophotometry, which estimates the absorption qualities of the visual pigment in isolated cones. Both methods have found a typical mammalian peak sensitivity of about 500 nm (Lythgoe 1972; Bowmaker 1991). The similar results from both methods suggests that the

optics of the tammar wallaby show no significant differential absorption at the recorded wavelengths. This is in agreement with the impression of a human observer that these structures are colourless. The ease with which the spectral sensitivity of the M-cones could be measured indicates that under the experimental conditions they dominated the responses.

It came as no surprise, therefore, that in the immunocytochemical study reported in chapter 3, the majority of cones could be labelled with an antibody raised against (human) M-cones. This study also showed that there is in fact a low density population of S-cones in the tammar retina. Both cones are present throughout the entire retina, but have very different spatial distributions. Based on these cone densities and the distribution of ganglion cells (Tancred 1981; Wong et al. 1986), it is possible to divide the retina of the tammar into three distinct regions. A dorsal region, with a balanced cone ratio (30% S-cones), which should provide a good differential spectral sensitivity and therefore acute colour vision, a central horizontal band of high acuity, and a ventral region with an unbalanced cone ratio (5% S-cones), which has a high overall cone but low ganglion cell density. This region, therefore, has a very high convergence ratio between cones and ganglion cells and is expected to have low acuity, but high sensitivity. It would be interesting to study behaviourally the spatial acuity, detection thresholds for small objects, temporal acuity and colour sensitivity in these regions of the retina, in order to test this hypothesis.

The immunocytochemical study also provided two possible explanations for the failure to find evidence for a functional S-cone population in the electrophysiological recordings: a) The S-cone density may have been too low to produce a measurable signal. In other words, the recording technique may not have been sensitive enough; b) An alternative explanation stems from the fact that the vast majority of S-cones could be double labelled with the antibody against M-cones and therefore may contain a significant proportion of the M-cone pigment. This is much more serious than just a low density. If this were the case, then any attempts to reduce the contrast seen by the M-cones during the recordings, in order to isolate the S-cone responses would also have reduced the contrast seen by the S-cones and therefore made it impossible to isolate their responses.

A behavioural study based on dual forced choice experiments (chapter 4) showed that the tammar wallaby has acute colour vision. It was demonstrated that the wallabies were able to discriminate colours just 3 nm apart. These experiments also confirmed the presence of S-cones in the tammar retina. Their peak spectral sensitivity was estimated to be about 420 nm, based on a series of Null-point estimates under different light conditions, which are essentially colour mixing experiments. Based on these same experiments, it is also possible to functionally characterise the tammar's colour vision as dichromatic. Evidence was also presented for the fact that the wallabies were able to use rod cone interactions in order to discriminate between two colours, when these same two colours could not be discriminated based on the two cone pigments alone.

In conclusion, the tammar wallaby has acute dichromatic colour vision, based on a 420 nm and a 540 nm cone pigment and a spatial acuity of about 5 c/deg. Its retina shows a differentiated topography, with three distinct regions, each hypothesised to be specialised for a particular task.

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